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Aproximación a la ecofisiología del milano negro ("Milvus Migrans"): efectos intrínsecos y socio-ambientales en la regulación de glucocorticoides y carotenoides

MEMORIA PARA OPTAR AL GRADO DE DOCTORA

PRESENTADA POR

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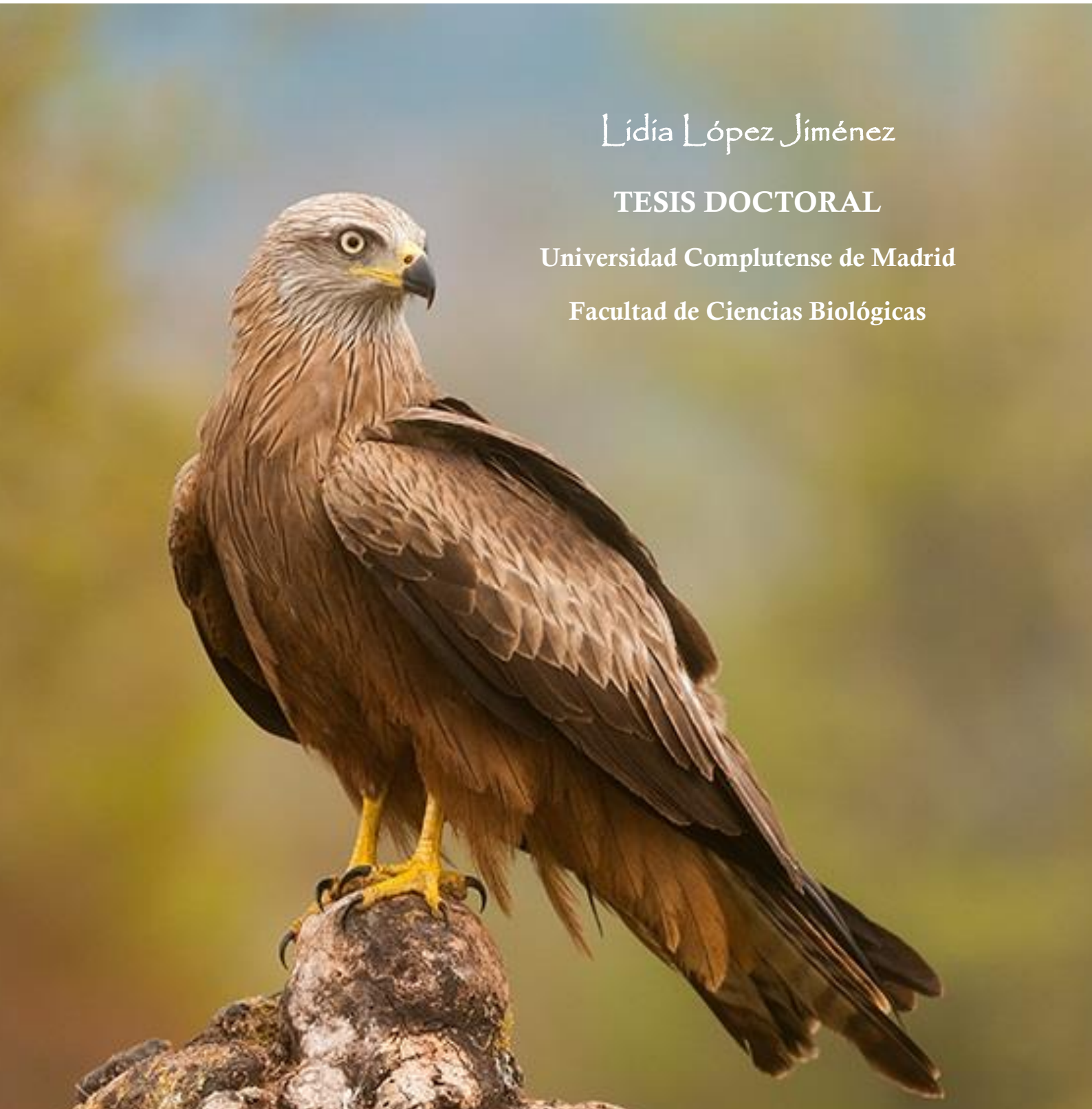
APROXIMACIÓN A LA ECOFISIOLOGÍA DEL MILANO NEGRO (*MILVUS MIGRANS*): EFECTOS INTRÍNSECOS Y SOCIO-AMBIENTALES EN LA REGULACIÓN DE GLUCOCORTICOIDES Y CAROTENOIDES



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A mis padres

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RESUMEN DE LA TESIS

La última década ha sido testigo de un auge en el número de estudios buscando esclarecer el modo en que los mecanismos fisiológicos facilitan las respuestas fenotípicas y conductuales a las condiciones ambientales bajo entornos naturales. Este reciente interés nació de la necesidad de trasladar las hipótesis y conclusiones generadas en el laboratorio a contextos más realistas, en donde los animales se ven expuestos a la influencia simultánea de múltiples factores y presiones selectivas. En este sentido, tanto los glucocorticoides como los carotenoides han venido recibiendo considerable interés, a razón de sus respectivos papeles como importantes mediadores fisiológicos de la homeostasis y procesos relacionados con la salud de los animales. No obstante, hasta la fecha, gran parte de las publicaciones relacionadas con el estudio de estos dos sistemas fisiológicos en aves silvestres han usado especies modelo de pequeño tamaño y corta vida (principalmente paseriformes), cuya ecología y vulnerabilidad ante los retos medioambientales difiere considerablemente de la de las aves de larga vida. En consecuencia, el objetivo de la presente tesis ha consistido en tratar de reducir esta brecha en el conocimiento mediante la aproximación a distintos aspectos de la ecofisiología de una rapaz salvaje, territorial, y extremadamente longeva (el milano negro, *Milvus migrans*). En concreto, los dos primeros capítulos ofrecen una evaluación en profundidad de los factores ambientales, sociales e intrínsecos que regulan la secreción de glucocorticoides (corticosterona; CORT) en pollos, usando para ello dos metodologías distintas para la determinación de los niveles de hormonas (plasma vs plumas; capítulos 1 y 2). Los capítulos 2 y 4, por el contrario, se centran en la porción adulta de la población. En el capítulo 3 se miden niveles de

CORT en plumas de individuos de todas las edades y etapas vitales (incluyendo ejemplares reproductores y no-reproductores), con el propósito de investigar, por primera vez, los cambios en la secreción de CORT a lo largo de toda la vida de una rapaz de larga vida, no-marina y no-colonial. Para terminar, el capítulo 4 profundiza en el efecto de las condiciones ambientales de fondo (en este caso los niveles de inundación de la marisma) sobre la regulación de los carotenoides plasmáticos y la esperada relación entre calidad individual e intensidad de coloración de los rasgos ornamentales basados en estos pigmentos.

En conjunto, las variaciones en los niveles de CORT en las plumas de los pollos mostraron que los principales retos a los que se enfrentan estas aves durante su periodo de desarrollo en el nido son las bajas temperaturas ambientales, la rivalidad entre hermanos y la escasez de alimento. Los niveles de CORT en plasma, por el contrario, mostraron una marcada ritmicidad diaria en la función adrenocortical, aumentos ontogénicos (tanto en los niveles basales como agudos de CORT) consistentes con la teoría del desarrollo, y elevaciones más robustas en respuesta al estrés de la captura en años de sequía. Estos resultados ponen de manifiesto la importancia de combinar varios métodos de cuentificación a fin de obtener una visión más completa del estado fisiológico de las poblaciones y de los retos más nocivos a los que se enfrentan los individuos. En adultos, los niveles de CORT en pluma exhibieron un descenso progresivo a lo largo de las primeras etapas de vida, alcanzando los niveles más bajos en individuos de mediana edad (7-11 años), pero aumentando de nuevo en edades posteriores. Curiosamente, el patrón observado exhibió, además, un notable paralelismo con los cambios en el éxito reproductor y la supervivencia relacionados con la edad en esta población, apuntando, por tanto, a un posible papel de CORT como principal determinante fisiológico de la eficacia biológica de estas aves a lo largo de toda su vida. Los

milanos, además, mostraron niveles de carotenoides plasmáticos significativamente elevados en años de mucha inundación comparados con años de sequía, lo que sugiere una mayor disponibilidad de estos pigmentos en el ambiente, posiblemente a través de comunidades más grandes de macrófitos y/o densidades de presas más altas. Al mismo tiempo, los altos niveles de inundación diluyeron por completo las diferencias en las concentraciones de carotenoides circulantes observadas entre no-reproductores y reproductores, y entre individuos en buena y mala condición física en años de sequía. Estos resultados evidencian la importancia de registrar las condiciones ambientales experimentadas por los individuos en el momento del muestreo a fin de comprender bien los mecanismos de regulación interna de estos pigmentos. Además, ofrecen una explicación plausible para las frecuentes discrepancias encontradas hasta la fecha entre distintos estudios. Por último, la variación inter-individual en la intensidad de coloración del tarso en años de mucha inundación mostró una fuerte asociación con la edad, similar a la observada anteriormente para el éxito reproductor, las probabilidades de supervivencia y los niveles de CORT en plumas en esta población. El hecho de que esta relación se observara en años en los que las diferencias en la calidad individual a nivel plasmático no se mostraron evidentes, sugiere que tanto las demandas internas como los mecanismos endógenos que regulan la incorporación de carotenoides al tegumento mantienen la honestidad de la información de la señal más allá de la disponibilidad de estos pigmentos, y apoya una posible función de estos rasgos en la comunicación intraespecífica.

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THESIS SUMMARY

The last decade has witnessed a surge in the number of studies aiming to unravel the way in which physiological mechanisms mediate the phenotypic and behavioural responses to environmental conditions under natural settings. Such recent interest for this approach stemmed from the recognized necessity to transfer the hypotheses and conclusions generated in the laboratory to more realistic contexts, in which animals experience the simultaneous effects of multiple interacting factors and selective pressures. In this sense, both glucocorticoids and carotenoids have received considerable attention so far, given their respective roles as important physiological mediators of homeostasis and health-related processes in animals. However, to date, much of the accumulated literature regarding these two physiological systems in wild birds has largely focused on small, short-lived species (mainly passerines), which differ greatly from long-lived birds both in terms of their general ecology and their vulnerability to environmental challenges. To shorten this gap in knowledge, the present thesis explores different approaches to the eco-physiology of a wild, long-lived, territorial raptor (the black kite, *Milvus migrans*). In particular, the first two chapters provide an in-depth assessment of the environmental, social and intrinsic factors that regulate endocrine secretion of glucocorticoids (corticosterone; CORT) in nest-bound nestlings, using two different methodologies for the determination of hormone levels (plasma versus feathers; chapters 1 and 2). Chapters 3 and 4, by contrast, focus on the adult-segment of the population. In chapter 3, feather CORT is measured in individuals of all ages and life-history stages (including both breeding and non-breeding sectors), with the purpose of investigating lifelong changes in CORT secretion for the first time in a long-lived, non-marine and non-colonial avian species. To finish, chapter 4 delves

into the effect of background environmental conditions (here exemplified by high and low marshland inundation levels) on the regulation of circulating carotenoids and the expected relationship between individual quality and intensity of carotenoid-based ornamentation.

Overall, variations in feather CORT levels revealed that black kite nestlings at the study population were most negatively affected by low ambient temperatures, sibling rivalry and food scarcity. Plasma CORT levels instead revealed marked diel rhythms in adrenocortical function, ontogenetic increases in both baseline and stress-induced CORT levels consistent with the developmental hypothesis, and stronger CORT rises in response to capture and restraint during drought years. These results highlight the importance of combining several methodologies to obtain a more complete picture of the physiological state of populations and of the factors that are most detrimentally challenging for individuals. In adults, feather CORT levels exhibited a gradual decline across the initial life-history stages, reaching lowest levels in prime-age individuals (7-11 years-old), but increasing again thereafter. Interestingly, the observed pattern mirrored the age-related changes in reproductive performance and survival previously reported for the same study population, thus pointing to a possible role of CORT as a major physiological determinant of fitness throughout life. Kites also exhibited elevated circulating carotenoid levels in years of high inundation compared to years of drought, possibly reflecting a higher availability of these pigments in the environment through larger macrophyte communities and/or higher prey densities. At the same time, high flood levels completely diluted the differences in plasma carotenoid concentrations observed between non-breeders and breeders, and between individuals in good and poor body condition in years of drought. The results thus highlight the importance of taking into account the full range of environmental conditions experienced by

individuals at the time of sampling in order to fully understand the mechanisms behind the regulation of carotenoid pigments, and provide a plausible explanation for the frequent discrepancies observed so far in the results reported by different studies. Finally, inter-individual variation in tarsus colour scores during wet years showed a strong association with age, similar to that observed for reproductive success, survival probabilities, migration performance and stress levels in this population. The fact that the relationship came out in years in which differences in individual quality at the plasma level were completely lacking suggests that both internal carotenoid demands, as well as the endogenous machinery responsible for the incorporation of carotenoids into the integument, actively maintain the honesty of the information conveyed by this bodily trait regardless of the availability of these pigments. Such finding lends considerable support to the idea that carotenoid-based coloured traits in black kites play a role in intraspecies communication.

INTRODUCCIÓN

La presente tesis doctoral es una contribución al conocimiento de la eco-fisiología de aves silvestres, y ha sido diseñada con la intención de integrar disciplinas científicas tradicionalmente separadas como son la ecología y la fisiología animal, utilizando un modelo de estudio poco convencional: la población de Milano negro (*Milvus migrans*) del Parque Nacional de Doñana. El estado actual de conocimiento en el campo de la fisiología en aves (y por extensión, en vertebrados), ha sido tradicionalmente sesgado por la experimentación en cautividad utilizando modelos animales fácilmente manipulables. Aunque esta base de conocimiento ha generado una relevante información (permitiendo, por ejemplo la formulación de nuevas hipótesis), numerosas conclusiones han sido tradicionalmente aceptadas obviando la ausencia de presiones selectivas reales e impidiendo la generalización de resultados a especies silvestres en su medio natural. Aunque es cierto que la comprobación de hipótesis fisiológicas en poblaciones de aves silvestres ha experimentado un auge durante las últimas décadas, no lo es menos que ha existido, y sigue existiendo un marcado sesgo hacia ciertos grupos (fundamentalmente paseriformes y aves marinas) como modelos de estudio, limitando la inferencia de resultados a otros grupos de aves. En contraposición, esta tesis aborda cuestiones fisiológicas relacionadas con el funcionamiento endocrino en respuesta al "estrés" (capítulos 1-3), los carotenoides plasmáticos y la expresión de señales (capítulo 4) enmarcándolas en un contexto ecológico y ambiental real, y ofreciendo un marco de trabajo novedoso en un modelo de fauna silvestre cuya historia vital está poco representada en trabajos de eco-fisiología. Además, en varios capítulos se han utilizado metodologías de muestreo de reciente evolución y poco invasivas, como es la determinación de corticosterona en muestras de pluma. Entre otros resultados, en esta tesis se analizan por primera vez los factores ambientales y sociales que afectan a la modulación de la corticosterona (principal

hormona del estrés) en pollos de aves rapaces, o cómo cambian los niveles de corticosterona a lo largo del ciclo vital completo en un ave de larga vida.

Durante el proceso de elaboración de esta tesis, he estado integrada en un equipo de trabajo multidisciplinar cuyo objetivo último es comprender la ecología del Milano negro en su medio natural. Al estar la población de estudio sujeta a un seguimiento científico mantenido durante décadas, el planteamiento de hipótesis, y la ejecución de los muestreos para esta tesis ha sido siempre simultaneado con tareas intensivas de seguimiento anual poblacional (p.ej. la captura y marcaje de pollos y adultos, el seguimiento reproductor individualizado), y la elaboración de trabajos científicos adicionales. A consecuencia, gran parte de mi producción científica durante el periodo de elaboración de esta tesis es el resultado de estos trabajos simultáneos, que no han sido incorporados al cuerpo de esta tesis al objeto de mantener un contenido eco-fisiológico homogéneo, pero que sin embargo han sumado 11 publicaciones adicionales en revistas indexadas. La tesis se ha estructurado finalmente en 4 capítulos, cuyo formato responde al de manuscritos independientes, al objeto de facilitar su publicación, o en el caso de los capítulos 1-3 porque ya están publicados o sometidos a evaluación por pares. Esta introducción, pretende presentar una visión más general de los temas de fondo tratados a lo largo de estos 4 capítulos, permitiendo explicar fuera del restringido formato de una publicación científica cuales son las hipótesis de trabajo, el estado actual de los temas de estudio, y los métodos de trabajo más frecuentemente utilizados. Además, incluye un apartado final explicando los objetivos generales que se han perseguido en cada uno de estos estudios.

Fisiología de los glucocorticoides

Los seres vivos en general, y por extensión las poblaciones de aves silvestres, se ven expuestos a continuos cambios espaciales y temporales en las condiciones ambientales de su entorno que amenazan potencialmente su supervivencia. En las aves, este riesgo viene en última instancia determinado por la capacidad de dichos cambios de alterar determinados sistemas fisiológicos que resultan esenciales (pH, temperatura corporal, niveles de glucosa en sangre y tensión de oxígeno arterial) y cuya estabilidad dentro de unos rangos óptimos relativamente estrechos (i.e. "*homeostasis"*), resulta imprescindible para el mantenimiento de la vida (revisado en Blas 2015). En consecuencia, las presiones selectivas han propiciado la evolución de mecanismos de respuesta que permiten al organismo realizar "*alostasis"*, es decir, mantener la homeostasis a través de cambios flexibles (McEwen & Wingfield 2003, Blas 2015). Para ello, determinados "mediadores alostásicos" fluctúan ampliamente y de forma paralela a las variaciones ambientales, logrando amortiguar el efecto sobre la homeostasis. Uno de los principales mediadores alostásicos es la respuesta adrenocortical, que conlleva una cascada de secreciones hormonales controladas por el eje hipotalámico-pituitario-adrenal (HPA), y que finaliza con la estimulación de la glándula adrenal y la subsecuente liberación de hormonas glucocorticoides (GCs: corticosterona en aves) en el torrente sanguíneo (revisado en Blas 2015). A su vez, una elevación en los niveles plasmáticos de GCs induce cambios adaptativos en el metabolismo, fisiología y conducta de los individuos, dirigidos a ayudar a los mismos a afrontar los desafíos que amenazan su homeostasis, fundamentalmente supliendo las demandas energéticas asociadas. Por ejemplo, diversos estudios en aves han demostrado que la secreción de corticosterona (CORT) actúa: (1) fomentando la actividad locomotora (lo que facilita al individuo a alejarse y eludir

la situación de riesgo; Breuner et al. 1998); (2) aumentando la tasa metabólica (ayudando, por ejemplo, a incrementar la producción de calor interno a medida que desciende la temperatura externa; Palokangas & Hissa 1971, Mujahid 2010, Wack et al. 2012); (3) estimulando la ingesta de alimentos (para incrementar las reservas internas de energía y poder hacer frente a los costes de la alostasis; Kitaysky et al. 2001, Kitaysky et al. 2003, Crossin et al. 2012); (4) facilitando la sumisión (evitando así posibles heridas o lesiones en enfrentamientos con rivales competitivamente superiores; Leshner & Politch 1979, Leshner 1980); (5) inhibiendo la síntesis de proteínas; (6) estimulando la gluconeogénesis (la síntesis de glucosa a partir de precursores no carbohidratos), la proteólisis y la movilización de las reservas corporales de grasa (con el fin de aumentar la disponibilidad de fuentes de energía inmediatas que sustenten las anteriores conductas; Sapolsky et al. 2000, Blas 2015); y (7) interrumpiendo el desarrollo de actividades y comportamientos no esenciales (ej. reproducción, defensa territorial) que permitan ahorrar energía (Wingfield & Silverin 1986; Angelier et al. 2009).

No obstante, cabe destacar que tanto la magnitud de la elevación en los niveles de GCs, como el tipo de respuesta suscitada pueden llegar a variar de manera considerable dependiendo de varios factores, entre los cuales destacan: (i) la naturaleza del desafío homeostático; (ii) su persistencia en el tiempo; y (iii) la "*carga alostática"* previa del individuo (McEwan & Wingfield 2003). Este último término hace referencia al total de energía requerido para "funcionar" en el día a día (i.e. para sustentar un metabolismo mínimo de mantenimiento, salir a buscar comida, termorregular, etc.), y que suele aumentar o disminuir de forma predecible según, por ejemplo, la estación del año (p. ej., más costes en invierno para afrontar las bajas temperaturas) o la etapa del ciclo vital en que se encuentra el individuo (p. ej., más demanda energética durante el desarrollo, la reproducción, la migración, etc.;

Landys et al. 2006, Wada 2008). A esto se suman, además, los costes adicionales en respuesta a las perturbaciones que tienden a surgir de manera impredecible a lo largo de toda la vida del animal, y que pueden ser puntuales y transitorios (p.ej., como resultado de interacciones agresivas entre conespecíficos, ataques de depredadores, tormentas pasajeras; Leshner 1980, Cockrem & Silverin 2002), o prolongados e incluso crónicos (p. ej. costes derivados de infecciones, lesiones temporales o permanentes, fenómenos climatológicos adversos prolongados como El Niño, etc.; Romero & Wikelski 2001). En conjunto, la interacción de todos estos factores constituye el entramado del marco teórico conocido como "*Modelo Alostásico"* (McEwen & Wingfield 2003), que a su vez ha generado otros modelos para explicar las fluctuaciones esperadas en los niveles de GCs (el "*Modelo Reactivo*"; Romero et al. 2009), sentando así las bases conceptuales para entender cómo los animales responden ante los distintos y continuos cambios ambientales y sociales de su entorno dependiendo del estado fisiológico en que se encuentran. Ambos modelos parten de la premisa de que todo desafío homeostático asociado a eventos o situaciones predecibles provoca un aumento en la carga alostática del individuo que puede ser anticipado, y que por lo tanto permite un ajuste progresivo de los sistemas fisiológicos. En estos casos, los niveles de GCs en plasma típicamente fluctúan poco y se mantienen dentro de unos márgenes relativamente estrechos que constituyen el *rango basal* o *predictivo*. Por el contrario, la experiencia de enfrentarse a perturbaciones o estímulos nocivos inesperados provoca en el animal una fuerte y rápida activación del eje HPA que estimula la secreción de grandes cantidades GCs, causando una elevación de los niveles plasmáticos más allá del rango predictivo, y dentro de un rango "*reactivo*". En la mayoría de ocasiones este tipo de respuesta es adaptativa, puesto que induce al individuo a modificar su fisiología y/o conducta para alejarse o afrontar la fuente de estrés en la

mejor condición posible hasta que esta pasa y deja de suponer un riesgo. Cuando esto ocurre, los niveles de GCs disminuyen rápidamente para volver a situarse dentro los límites del rango predictivo, permitiendo así al animal reasumir de nuevo sus actividades cotidianas. Un aspecto importante a resaltar es que no todas las perturbaciones producen la misma intensidad de respuesta hormonal, ni todos los individuos responden igual ante un mismo estímulo. No obstante, la respuesta en todos los casos se considera adaptativa siempre y cuando los niveles máximos de GCs en plasma no sobrepasen el umbral superior de lo que se conoce como el *rango reactivo* de cada individuo, ya que por encima de él el animal entra en un estado de *sobrecarga homeostática* en el que los altos niveles de GCs pueden comenzar a generar graves patologías (Romero et al. 2009). El problema, en este sentido, surge cuando en ciertas situaciones o épocas señaladas del año, la carga alostática que soporta un individuo se ve considerablemente incrementada (p. ej., durante la época de reproducción o durante un proceso infeccioso), provocando una elevación en los niveles basales de GCs cerca del límite del rango predictivo (Kitaysky et al. 1999, Kitaysky et al. 2001b, Williams et al. 2008). Así, una misma intensidad de respuesta alostática frente a una perturbación ambiental o social puede producir resultados muy distintos (adaptativos vs perjudiciales) dependiendo del estado fisiológico previo del animal (i.e. a menor carga alostática previa, menores niveles basales de GC y más amplio el margen de respuesta aguda antes de incurrir en una sobrecarga homeostática). Por otro lado, cabe también la posibilidad de que una respuesta en principio adaptativa se convierta en patológica a largo plazo. La razón de ello radica en que unos niveles de GCs agudos y sostenidos en el tiempo, aun estando dentro del rango reactivo (adaptativo) del individuo, generan un coste que a la larga provoca un "desgaste" fisiológico. Este desgaste (consecuencia de invertir prioritariamente la energía disponible en mecanismos asociados a la respuesta

alostática, en detrimento de otras tareas esenciales como las de mantenimiento y reparación celular) merma la capacidad del animal para afrontar nuevas perturbaciones, lo que provoca, a su vez, un descenso gradual del umbral del rango reactivo. Así pues, los niveles de GCs en sangre (incluso cuando se mantienen estables) pueden verse cada vez más cerca del rango de sobrecarga homeostática, llegando incluso al punto de entrar en él si la fuente de estrés no cesa o desaparece. Este tipo de situaciones puede darse en casos de animales salvajes expuestos a la presión continua de depredadores, en individuos que sufren alguna lesión o enfermedad prolongada, o bien en grupos sociales en los que los individuos subordinados se ven sometidos a constantes agresiones e intimidaciones por parte de conespecíficos de mayor rango dentro de la escala jerárquica (Goymann & Wingfield 2004, Lobato et al. 2008, Blas et al. 2011, Bryan et al. 2015). En todos estos escenarios, la capacidad del individuo de reconducir la situación y reducir la carga alostática es de vital importancia para su supervivencia, dado que la secreción aguda y crónica de GCs puede producir múltiples efectos adversos y, eventualmente, causar la muerte. Gran parte de nuestros conocimientos hasta la fecha sobre este aspecto provienen de estudios experimentales en aves, donde los sujetos son implantados con una pequeña cápsula que libera CORT en el organismo durante semanas, simulando así la exposición a una fuente de estrés crónica. De este modo, se ha podido demostrar que en este tipo de situaciones, los niveles de CORT puede llegar a: (1) reducir la tasa de crecimiento (lo que puede acarrear consecuencias en la eficacia biológica a largo plazo; Müller et al. 2009, Fairhurst et al. 2013); (2) deprimir el funcionamiento del sistema inmunitario (aumentando la vulnerabilidad a sufrir infecciones; Bourgeon & Raclot 2006); (3) afectar negativamente el desarrollo cognitivo del animal (Kitaysky et al. 2003); (4) reducir la supervivencia (Kitaysky et al. 2001a); (5) disminuir la tasa de crecimiento

y la calidad de las plumas mudadas durante el periodo de estrés crónico (Romero et al. 2005, DesRochers et al. 2009); o (6) producir una pérdida de masa muscular (Gray et al. 1990).

Función adrenocortical durante el desarrollo

La fase del desarrollo embrionario y post-embrionario es una de las más sensibles del el ciclo vital animal, siendo este el periodo de mayor vulnerabilidad a los cambios y agresiones externas. A pesar de ello, los vertebrados en general, y las aves en particular, muestran gran variabilidad de estrategias vitales en esta fase, lo que condiciona importantes fluctuaciones en el grado de exposición a los desafíos ambientales. En aves, las estrategias de desarrollo abarcan un gradiente comprendido entre modos altriciales y precociales de desarrollo (Gill 2006). En el extremo altricial se encuentran las especies cuyos pollos nacen en un estadio muy prematuro de desarrollo, completamente desnudos, ciegos, incapaces de termorregular, con escasa movilidad y nula habilidad locomotriz, por lo que dependen totalmente de sus padres para conseguir alimento, mantener la temperatura corporal y resistir las inclemencias del tiempo o intentos de depredación. En el extremo opuesto, las aves precociales nacen en un estadio muy avanzado de desarrollo, emplumadas, con los ojos abiertos, y capaces de termorregular, andar y buscar comida por sí mismas, aunque requieren también de la protección parental frente a posibles depredadores. Entre estos dos extremos se encuentran las aves semi-altriciales y semi-precociales, que nacen con una capa de plumón, los ojos abiertos (o al cabo de unos pocos días) y distintos grados de movilidad, pero aun incapaces de termorregular de forma adecuada. Teóricamente,

la incapacidad de resistir retos homeostásicos debería convertir a los pollos no-precociales en sujetos sumamente susceptibles de sufrir elevaciones en los niveles plasmáticos de GCs, con el consiguiente riesgo para su salud. No obstante, en la práctica, la capacidad para activar el eje HPA en respuesta a una perturbación tiende a mostrar una fuerte asociación con la adquisición de habilidades y capacidades, y por tanto con el modo y el estadío de desarrollo ontogénico (Blas & Baos 2008). Así pues, mientras que los pollos de especies precociales son capaces de exhibir una respuesta similar a la de los adultos (en cuanto a rapidez y magnitud) a las pocas horas de eclosionar (Holmes et al. 1990, Jenkins & Porter 2004), los pollos de las especies no-precociales (i.e. altriciales, semi-altriciales y semi-precociales) suelen experimentar un periodo hipo-sensible tras la eclosión, caracterizado por una respuesta atenuada o nula al estrés, seguido de un aumento progresivo en la intensidad de su respuesta con la edad (Sims & Holberton 2000, Blas et al. 2006a, Wada et al. 2007, Müller et al. 2010). De acuerdo con la "Hipótesis del Desarrollo" planteada por Sims y Holberton (2000), este patrón reflejaría el modo en que los animales sincronizan la maduración o activación de las glándulas del eje HPA con el desarrollo físico, psíquico y fisiológico de las habilidades y mecanismos que les permiten realizar los necesarios ajustes para afrontar las posibles perturbaciones, consiguiendo así alcanzar un compromiso entre los efectos adaptativos de la secreción aguda de GCs y los efectos nocivos del estrés crónico.

Con respecto a los niveles de CORT basales, estudios en especies precociales como la gallina (*Gallus domesticus*), muestran que la secreción endógena de CORT se inicia en las últimas etapas embrionarias, jugando un papel importante en el desarrollo final de los órganos vitales (p.ej., los pulmones; Hylka & Doneen 1983) y estimulando la propia eclosión (revisado en Wada 2008 y Blas 2015). Por

desgracia, hasta la fecha nada se sabe del momento exacto en el que el eje HPA comienza a producir CORT en aves altriciales y no-precociales, aunque Schwabl (1999), Sims & Holberton (2000) y Sockman & Schwabl (2001) reportaron niveles detectables de esta hormona en pollos de 5 días de canario (*Serinus canaria*), sinsonte norteño (*Mimus polyglottos*) y cernícalo americano (*Falco esparverius*), respectivamente. Estos resultados sugieren, como mínimo, que el eje HPA adquiere cierto grado de funcionalidad poco tiempo después de la eclosión, reforzando a su vez la idea de que las variaciones en los niveles de GCs, dentro del rango predictivo, son vitales para la regulación de procesos internos asociados a la homeostasis incluso en pollos. En este sentido, influye el hecho de que el desarrollo de un ave constituye un periodo de elevada inversión y demanda energética, pues a la energía requerida para el mantenimiento de las funciones metabólicas básicas ha de sumarse una gran cantidad de energía adicional para la formación de huesos, masa muscular y otros tejidos (p. ej., en el caso de paseriformes, el desarrollo debe completarse en tan sólo un par de semanas).

Cuantificación de glucocorticoides

En las últimas décadas, el campo de la eco-fisiología ha vivido un importante avance en el desarrollo de técnicas que permiten la medición de niveles de GCs en distintos tipos de muestras biológicas, permitiendo obtener estimas del estado fisiológico de individuos o poblaciones silvestres en relación a una multitud de factores ambientales, sociales, físicos y fisiológicos (Wingfield et al. 1997, Blas et al. 2015). Cada una de estas técnicas ofrece sus pros y sus contras, y por tanto la decisión de cual emplear debe ser valorada en base a un conjunto de criterios, entre

los que se incluyen: (i) el tipo de estudio a desarrollar (ej. campo vs laboratorio); (ii) la especie modelo (ej. ave vs mamífero; grande vs pequeña; abundante vs en peligro); y (iii) el tipo de información que desea obtenerse (ej. largo plazo vs corto plazo). En estudios con aves, prevalece la cuantificación de CORT en plasma frente al uso de otros sustratos menos invasivos, como son las muestras fecales o las plumas. La determinación de GCs en plumas fue propuesta y desarrollada hace menos de una década (Bortolotti et al. 2008), y está ganando creciente interés en estudios de campo gracias a su potencial para ofrecer una medida integrada y a largo plazo de la actividad adrenocortical de un individuo. A continuación, se ofrece un resumen de las dos técnicas empleadas en los capítulos de esta tesis doctoral (medición en sangre y pluma), con particular atención a la metodología, el tipo de información que aporta cada una de ellas y sus principales restricciones.

Corticosterona en plasma

El desarrollo, hace ya 4 décadas, de una técnica para medir concentraciones de hormonas esteroides en volúmenes pequeños de sangre mediante radioimmunoensayo (RIA; Wingfield & Farner 1975) abrió la puerta a la posibilidad de obtener muestras de sangre seriadas de un mismo individuo sin necesidad de sacrificarlo, marcando así el inicio de la disciplina conocida como endocrinología de campo (Wingfield & Farner 1976). Uno de los valores a destacar de esta técnica es que permite cuantificar por separado los niveles basales y agudos de CORT en plasma, ofreciendo de este modo valiosa información sobre la carga alostática de un individuo en el momento del muestreo (en el caso de los niveles basales), además del grado de activación de su eje HPA ante un desafío inesperado (en el caso de los niveles agudos). Para ello, existe un protocolo muy extendido en

estudios de endocrinología (el "protocolo de captura y restricción"; Wingfield & Farner 1976; Blas 2015), que consiste en someter a un sujeto al estrés estándar de la captura e inmovilización, y tomarle repetidas muestras de sangre a distintos intervalos de tiempo prefijados (por ejemplo: 0, 10, 30 y 60 minutos; Wingfield et al. 1997). El objetivo de la primera muestra en estas series de estrés es conseguir una medida de los niveles basales de CORT del animal (i.e. dentro del rango predictivo). Sin embargo, para que esta sea válida, ha de obtenerse necesariamente dentro de los primeros 2-3 minutos post-captura (p. ej., a contar desde el momento en que el individuo es capturado), ya que después de este tiempo los niveles de CORT en plasma comienzan a aumentar rápidamente (Romero & Romero 2002, Romero & Reed 2005). El resto de muestras seriadas, a su vez, sirven para caracterizar el perfil de activación adrenocortical del individuo, siendo imprescindibles al menos dos parámetros: (i) la concentración máxima y/o el incremento máximo de CORT (i.e. la diferencia entre los niveles agudos y los niveles basales); y (ii) la rapidez de respuesta (i.e. el tiempo que tarda en alcanzar los niveles máximos).

Otro punto fuerte de esta técnica es que permite valorar el estado fisiológico del animal en relación a las condiciones ambientales presentes en el momento en que se toma la muestra (Wingfield et al. 1983, Romero et al. 2000, Jenni-Eiermann et al. 2008). Además, permite también evaluar cambios en los niveles de CORT y en la capacidad de respuesta al estrés de un mismo individuo a lo largo de distintas escalas temporales (p. ej., durante el corto periodo de desarrollo en el nido, a través de las estaciones o a lo largo de los años; Love et al. 2003, Ouyang et al. 2011, Rensel & Schoech 2011). La utilización de muestras de sangre, no obstante, resulta un método relativamente invasivo, lo que puede generar riesgos y controversia, especialmente cuando se trabaja con especies amenazadas. Además, las muestras

requieren condiciones especiales de conservación (frío) y un procesamiento previo a la extracción de corticosterona (centrifugación y separación del plasma de la fracción celular), lo que a veces resulta inconveniente si el muestreo se lleva a cabo en el campo y especialmente en sitios remotos. Por otra parte, en este tipo de estudios de campo a menudo se desconocen los acontecimientos acaecidos momentos antes de la captura del ave, por lo que si un individuo, por ejemplo, sufre un episodio estresante en los minutos u horas previas, su perfil adrenocortical podría verse alterado y sus niveles de CORT posiblemente elevados fuera del rango predictivo en el momento de tomar la muestra basal.

Corticosterona en pluma

Durante el crecimiento de una pluma, el folículo dermal se vasculariza con el fin de aportar nutrientes a los procesos activos de proliferación y diferenciación celular. Dicha vascularización implica que todo tipo de moléculas lipofílicas presentes en el torrente sanguíneo, incluida la corticosterona, queden depositadas en la matriz queratinosa de la pluma a medida que esta se va formando, y en cantidades proporcionales a su concentración en plasma (Bortolotti et al. 2008, Jenni-Eiermann 2015). Sin embargo, una vez que la pluma termina de crecer, el riego sanguíneo desaparece dejando una estructura inerte, incapaz de incorporar nuevas moléculas desde la circulación. Estas dos premisas instigaron recientemente el desarrollo de una técnica para extraer y cuantificar los niveles de CORT en plumas, partiendo de la hipótesis de que una vez crecidas, estas estructuras servirían como registro histórico de la actividad adrenocortical del individuo durante el periodo relativamente amplio (entre días y semanas) que abarcó su crecimiento (Bortolotti et al. 2008). Desde entonces, un creciente número de

estudios ha permitido validar la técnica y valorar su potencial como herramienta para identificar, de forma rápida y fiable, posibles factores adversos para la salud y supervivencia de individuos y poblaciones. Hasta la fecha, la técnica ha sido usada con éxito en alrededor de 45 especies de aves (todas en las que se ha probado) pertenecientes a varios taxones, siendo algunos de ellos filogenéticamente distantes entre sí (p. ej., Bortolotti et al. 2009, Carrete et al. 2013, Fairhurst et al. 2013, Kouwenberg et al. 2013). Además, se han demostrado relaciones entre los niveles de CORT en plumas y múltiples factores socio-ambientales e intrínsecos, tales como la condición corporal (Crossin et al. 2013, Legagneux et al. 2013, Will et al. 2014), diferentes parámetros reproductivos (Bortolotti et al. 2008, Crossin et al. 2013, Kouwenberg et al. 2013), la supervivencia en pollos (Fairhurst et al. 2013), el estatus social (Bortolotti et al. 2008), la distancia migratoria (Bourgeon et al. 2014), el tamaño del área de campeo (Carrete et al. 2013), la climatología y estacionalidad (Bortolotti et al. 2008; Fairhurst et al. 2012; Legagneux et al. 2013) y la expresión de caracteres externos basados en carotenoides (Lendvai et al. 2013, Martínez-Padilla et al. 2013).

La cuantificación de GCs en plumas ofrece numerosas ventajas metodológicas y logísticas. En primer lugar, se trata de un método de muestreo poco o nada invasivo (las plumas pueden incluso recogerse del suelo después de ser mudadas), y el sustrato de estudio no requiere medidas especiales para su preservación (pueden conservarse a temperatura ambiente; Bortolotti et al. 2009). En cuanto al tipo de información que aporta, los niveles de CORT ofrecen una estima integrada y a más largo plazo de la carga alostática de un individuo durante un periodo concreto, reflejando tanto las fluctuaciones experimentadas en los niveles basales de CORT, como la frecuencia y duración de la activación aguda del eje HPA en respuesta a situaciones de estrés (Bortolotti et al. 2008, Lattin et al.

2011, Jenni-Eiermann et al. 2015). La técnica, además, ofrece varias posibilidades desde el punto de vista metodológico. Por ejemplo, dividiendo la pluma en distintos segmentos y analizándolos por separado se consigue estudiar posibles cambios en la secreción de CORT a lo largo de diferentes periodos del crecimiento de la misma, logrando de este modo obtener información a distintas escalas temporales (Bortolotti et al. 2009, Will et al. 2014, Jenni-Eiermann et al. 2015). También es posible inducir el crecimiento de plumas nuevas fuera del periodo de muda natural para así obtener y comparar los perfiles de activación adrenocortical en distintos momentos del año (siempre y cuando la recaptura sea factible; Bortolotti et al. 2008).

Por último, cabe mencionar algunas de las restricciones que han de tomarse en cuenta si se desea optar por este método de análisis. La primera es que en estudios con aves salvajes puede ser muy difícil conocer el momento preciso en que creció la pluma, dificultando establecer una relación entre los niveles de CORT y las condiciones ambientales, sociales o intrínsecas experimentadas en ese periodo. Otra desventaja del uso de plumas, es que tampoco permite separar la contribución relativa de las fluctuaciones hormonales dentro del rango predictivo de aquellas que abarcaron el rango reactivo (se miden niveles totales de CORT). Consecuentemente, no es posible distinguir si unos niveles elevados de CORT en pluma son el resultado de muchos episodios estresantes pero breves o, por el contrario, de un único episodio prologado. Para acabar, es importante conocer bien la biología de la especie de estudio a la hora de interpretar los resultados. En concreto, es imprescindible saber cómo afectan las variaciones en la condición corporal a la tasa de crecimiento de las plumas y su densidad, ya que estos dos factores pueden alterar significativamente la cantidad de CORT depositada (Bortolotti et al. 2009, Jenni-Eiermann et al. 2015, Patterson et al. 2015).

Fisiología de los carotenoides

Los carotenoides son moléculas pigmentarias de origen no animal, naturaleza lipofílica y amplio abanico de funciones para animales y plantas (McGraw 2006). Entra otras, los carotenoides son los pigmentos responsables de producir muchas de las coloraciones amarillas, naranjas y rojas que se observan en la naturaleza. En aves, los carotenoides son responsables de gran parte de las coloraciones brillantes visibles en estructuras tegumentarias, incluidas plumas, piel, pico y cera. Aunque la mayoría de especies circulan cantidades variables de carotenoides en plasma, no todas exhiben este tipo de coloración, sugiriendo que el proceso por el que las moléculas se incorporan a este tejido es altamente selectivo y ocurre bajo un estricto control fisiológico (revisado en McGraw 2006). Los carotenoides también desempeñan un papel importante en las capacidades visuales de las aves y como fotoprotectores de los tejidos corporales expuestos a la radiación UV (Cuthill 2006, Blount & Pike 2012). Finalmente, aunque no menos importante, los carotenoides poseen funciones de salud, en virtud de múltiples propiedades inmunoestimulantes, tales como promover la proliferación de linfocitos, aumentar la actividad de macrófagos y células T citotóxicas, o estimular la liberación de citoquinas e interleucinas (Bortolotti et al. 2000, Saino et al. 2003, Blas et al. 2006b). Además, poseen una estructura molecular con gran capacidad para aceptar electrones libres, por lo que se les ha atribuido una importante función antioxidante y protectora frente al efecto nocivo de radicales libres en el organismo (von Schantz et al. 1999, McGraw et al. 2006). En consecuencia, son muchas las funciones que se disputan el uso de los carotenoides. Sin embargo, sólo las plantas, las algas y algunas bacterias son capaces de sintetizar estos pigmentos. Los animales, en

cambio, deben por fuerza obtenerlos y acumularlos a través de la dieta, lo que suele dar lugar a marcadas diferencias inter-específicas e inter-individuales en el grado de acceso a estos recursos, principalmente en función de: (1) el tipo de dieta (menor acceso cuanto más carnívora, puesto que la concentración de carotenoides en los tejidos corporales se diluye en cada eslabón de la cadena trófica; Bortolotti et al. 2000, Sternalski et al. 2010); y (2) la habilidad de cada individuo para encontrar y adquirir los pigmentos (Casagrande et al. 2006). Ante estas limitaciones, los individuos deben enfrentarse a la decisión de invertir preferentemente los carotenoides disponibles en funciones que aumenten sus probabilidades de supervivencia (i.e. inmunoestimulación/oxidación), o bien en aquellas que aumenten sus probabilidades de apareamiento (i.e. ornamentación). Esta disyuntiva motivó la idea de que las diferencias en la intensidad de coloración entre individuos podrían actuar como señales honestas de la calidad del portador, suponiendo que sólo los individuos sanos, además de aquellos con las suficientes habilidades para conseguir acumular grandes cantidades de carotenoides de la dieta, pudiesen permitirse destinar grandes cantidades de pigmento al tegumento (Lozano 1994). A nivel empírico, la hipótesis ha recibido considerable apoyo de estudios que han logrado relacionar con éxito el nivel de infección, el desarrollo de una respuesta inmunitaria ante un desafío experimental, o una tasa inferior de consumo de invertebrados, con una menor intensidad de coloración (Negro et al. 2000, Hõrak et al. 2001, McGraw & Ardia 2003, Casagrande et al. 2006, Baeta et al. 2008).

OBJETIVOS

Las condiciones ambientales y sociales a las que se enfrenta un individuo en su día a día tienen la capacidad de inducir cambios significativos en su conducta, fisiología e incluso su morfología, llegando, en última instancia, a afectar también a su supervivencia (Wikelsky & Thom 2000, Sergio 2003). A pesar de ello, los mecanismos fisiológicos responsables de regular estos cambios apenas se están empezando a comprender. En este sentido, tanto los glucocorticoides como los carotenoides han suscitado un creciente interés en las últimas décadas debido a su posible papel como agentes mediadores en estas asociaciones. Hasta la fecha, muchos estudios han logrado demostrar con éxito cómo diversos factores socio-ambientales e intrínsecos afectan a la función adrenocortical y a la regulación de los carotenoides (Ramos-Fernández et al. 2000, Hõrak et al. 2001, Lobato et al. 2008, Casagrande et al. 2009). No obstante, existe un claro sesgo en el número de estos enfocados hacia especies precociales, altriciales de corta vida (e.j. paseriformes) o aves marinas coloniales. Por el contrario, relativamente poco se sabe de la regulación de GCs y carotenoides en rapaces, a pesar de que estas aves muestran una ecología totalmente distinta a la de las anteriores. En consecuencia, la presente tesis parte del objetivo global de tratar de explorar la generalidad de los resultados de anteriores estudios, usando como modelo de estudio una rapaz territorial de larga vida (el milano negro, *Milvus migrans*). Esta especie posee una de las mayores distribuciones (en cuanto a aves rapaces se refiere) a nivel global, estando presente en gran parte de las zonas templadas y tropicales de todo el Paleártico, Indo-Malasia y Australasia (Cramp & Simmons 1980, Blanco & Viñuela 2004). En España, la población de milano negro está cifrada en torno a 10,000 parejas reproductoras, lo que la sitúa como la segunda mayor de Europa, con Rusia a la cabeza (Cramp & Simmons 1980, Blanco y Viñuela 2004, Palomino 2006). No obstante, detrás de este buen dato nacional se esconde un continuo y alarmante

descenso de más del 10% de la población en los últimos 18 años, por lo que la especie se encuentra actualmente catalogada como "Casi Amenazada (NT)" en el Libro Rojo de las Aves de España (Blanco y Viñuela 2004). A este problema se suma el hecho de que es una rapaz migratoria, cuyos cuarteles de invernada se sitúan en el Sahel, al sur del desierto del Sahara, principalmente en países occidentales como Mauritania y Mali (Sergio et al. 2014), en ecosistemas periódicamente afectados por fuertes sequías y donde las políticas de conservación probablemente ejercen poca influencia. Por todo esto, el segundo objetivo de la tesis, más enfocado a la conservación, persigue también profundizar en estos dos aspectos fisiológicos a fin de ayudar a entender mejor los factores que influyen más negativamente en el estado de salud de estas aves.

En concreto, los capítulos 1 y 2 analizan el efecto de las variaciones en una serie de variables ambientales, sociales e intrínsecas (p.e.j. sexo, condición corporal) sobre los niveles de glucocorticoides (CORT) en pollos. Los pollos de milano, al igual que los del resto de rapaces, son semi-altriciales. Es decir, nacen con una fina capa de plumón y con los ojos abiertos, pero incapaces de termorregular, andar o alimentarse por sí solos. Estás características podrían inducir una atenuación de la respuesta aguda al estrés durante las primeras etapas del desarrollo, así como un aumento progresivo en la intensidad de activación adrenocortical a medida que los pollos adquieren las habilidades necesarias para afrontar las posibles perturbaciones, logrando de este modo reducir el riesgo de incurrir en una sobrecarga homeostática (Sims & Holberton 2000). Sin embargo, los pollos de milano, a diferencia de los paseriformes, exhiben un comportamiento muy agresivo entre hermanos desde los primeros días de vida. Estas peleas, que suelen consistir en picotazos, intimidaciones, e incluso asfixia por aplastamiento (Viñuela 1999), parecen tener como objetivo establecer una jerarquía de acceso a la comida que

aportan los padres, ya que por lo general tienden a agravarse en situaciones de escasez de alimento, y a desaparecer alrededor de la tercera semana de vida, cuando los hermanos finalmente "asumen" su respectivos roles (dominante o subordinado; Drummond & Osorno 1992, Viñuela 1999). En este contexto, los pollos de milano podrían necesitar un eje HPA más desarrollado que el de otras aves altriciales al nacer, a fin de facilitar la expresión de respuestas adaptativas que les permitan hacer frente a este tipo de desafíos ineludibles a la escala del nido (p. ej., permitiendo la movilización de las reservas de grasas, Sapolski et al. 2000; o los comportamientos de sumisión, Leshner 1980). Por este motivo, el segundo objetivo de estos dos capítulos será el de explorar patrones ontogenéticos en la función adrenocortical durante el desarrollo, así como las diferencias en la carga alostática que soportan los hermanos mayores (dominantes), pequeños (subordinados) y los pollos únicos. Para llevar a cabo todo esto, se emplearán dos métodos distintos de cuantificación de glucocorticoides (plasma y pluma), permitiendo comparar el tipo de información que genera cada uno y valorar la importancia del uso simultáneo de técnicas complementarias en estudios eco-fisiológicos.

Los siguientes dos capítulos (3 y 4) se centrarán en la población adulta de milano negro. El capítulo 3 tiene como objetivo analizar las variaciones en los niveles de CORT en pluma de estas aves a lo largo de todas las etapas de su vida, permitiendo inferir el papel que los procesos de aprendizaje y la escalada en el status social ejercen sobre la carga alostática. Por último, el capítulo 4 estará dedicado a la regulación de los carotenoides en plasma y tegumento. El milano negro es una rapaz generalista pero estrictamente carnívora. En consecuencia, cabe esperar que su ingesta de carotenoides sea limitada y muy por debajo de su máximo fisiológico, al igual que se ha demostrado anteriormente en otras aves de presa mediante estudios experimentales de suplementación con estos pigmentos

(Casagrande et al. 2007, De Neve et al. 2008, Sternalski et al. 2010). En teoría, dicha limitación debería resultar en fuertes diferencias entre individuos de alta y baja calidad en los niveles plasmáticos de carotenoides y en la intensidad de la coloración de los rasgos ornamentales, ya sea porque los primeros son capaces de seleccionar presas con alto contenido en pigmentos, porque tienen una tasa más alta de éxito de capturas, o porque poseen un sistema inmunitario más eficiente. No obstante, en la práctica existen múltiples discrepancias en los resultados aportados por distintos estudios en torno a estas asociaciones, lo que por el momento ha impedido obtener una idea clara e inequívoca de los mecanismos que regulan los niveles de carotenoides en estas y otras aves. La razón de esto podría guardar relación con el hecho de que, en muchos casos, las condiciones ambientales de fondo no son tenidas en cuenta, a pesar del reconocido impacto que estas pueden ejercer en diversos aspectos fisiológicos, incluida la regulación de carotenoides (No & Storebakken 1991, Vergara et al. 2012a). Por ejemplo, bajo entornos favorables, caracterizados por ambientes poco competitivos y de gran abundancia de recursos alimenticios, podría resultar relativamente fácil para la mayoría de individuos acumular grandes cantidades de estos pigmentos, por lo que las diferencias a nivel plasmático entre individuos de alta y baja calidad podrían verse diluidas (Vergara et al. 2012b). Siguiendo, pues, esta línea de argumentación, y con el fin de aportar una explicación plausible para las frecuentes inconsistencias entre estudios previos, el último capítulo se dividirá en tres partes, cuyos objetivos respectivos serán: (1) determinar si distintas calidades de hábitat (en términos de inundación de la marisma) pueden influir de manera directa en los niveles plasmáticos de carotenoides de milanos; (2) explorar si la relación entre los niveles de carotenoides en plasma y varios indicadores de calidad individual y estatus social difieren bajo distintos contextos ambientales; y, por último, (3) investigar si la coloración de las

patas es capaz de mantener su honestidad como indicador de la calidad de los individuos incluso en años favorables, en los que las diferencias interindividuales en los niveles de carotenoides en plasma caben esperarse mínimas.

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CAPÍTULO 1

Effects of ontogeny, diel rhythms and environmental variation on the adrenocortical physiology of semi-altricial black kites (*Milvus migrans*)

Effects of ontogeny, diel rhythms and environmental variation on the adrenocortical physiology of semi-altricial black kites (*Milvus migrans*)

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ABSTRACT

We examined whether hypothalamic-pituitary-adrenal axis activity in the nestlings of a semi-altricial raptor, the black kite (*Milvus migrans*), varied with advancing age, throughout the day and in response to a number of socio-ecological factors presumed to affect allostatic load. Both baseline corticosterone (CORT) titers and maximum CORT levels during 30 minutes of handling and restraint augmented across all sampled ages, suggesting that nestlings' energetic demands and capacity to respond to perturbations increase progressively throughout development. CORT secretion also peaked in the early morning, reached minimum levels in the central hours of the day and increased again before dusk, suggesting a possible role of CORT in the regulation of activity-inactivity patterns. Finally, nestlings raised in a year of low marsh inundation, implying lower food availability and heightened allostatic loads, exhibited higher adrenocortical responsiveness to stress than nestlings raised in years of intermediate or high flooding. The non-detectable effect of other socio-ecological variables, such as territory quality, temperature or brood order, suggests that parents may be able to buffer their nestlings from adverse environmental conditions, or that the effect of such factors may have been obscured by selective mortality operating before sampling. We propose that future studies increase the simultaneous use of complementary techniques (fecal sampling, feather analysis) to reach firmer and more comprehensive conclusions, especially for planning the management of conservation sensitive species.

INTRODUCTION

Free-living animals are inevitably exposed to ever-changing environmental conditions that challenge stability in internal systems (i.e. homeostasis). In response, vertebrates have evolved physiological mechanisms that mediate the necessary allostatic adjustments to deal with these fluctuations and thrive (McEwen and Wingfield 2003; Romero et al. 2009). One such mechanism involves the hypothalamic-pituitary-adrenal (HPA) axis, which regulates secretion of glucocorticosteroids (GCs) into the bloodstream (reviewed in Blas 2015). GCs comprise a family of steroid hormones that facilitate coping with the extra energy demands associated with both predictable and unexpected challenges by inducing changes in physiology and behavior aimed at keeping or returning overall energy requirements (i.e. the allostatic load) below energy input levels (McEwen and Wingfield 2003). For example, short-term rises in plasma corticosterone (CORT, the main GC in birds) have been shown to promote (a) locomotion (to move away and escape from noxious stimuli; Breuner et al. 1998), (b) nestling begging (to encourage parental provisioning rates; Kitaysky et al. 2001; Kitaysky et al. 2003), (c) foraging and food intake rates (to increase energy input; Kitaysky et al. 2003; Crossin et al. 2012), (d) mobilisation of fat and glucose stores (to boost the energy supply to cells; Sapolsky et al. 2000), (e) suppression of territoriality/reproduction (to save energy by halting investment in non-essential behaviors; Wingfield and Silverin 1986; Angelier et al. 2009), or (f) thermogenesis (to adjust body temperature; Palokangas and Hissa 1971; Mujahid 2010), among others.

While the above responses are clearly adaptive, long-term sustained CORT elevations can, on the contrary, incur severe fitness costs on the individual in terms

of reduced growth, immune function, cognitive abilities, future recruitment and survival probabilities, as well as increased oxidative damage (Kitaysky et al. 2003; Blas et al. 2007; Elftman et al. 2007; Müller et al. 2009; Constantini et al. 2011). Such might be the case in situations where the perturbation extends in time (e.g. prolonged food shortage or permanent risk of predation), or where conditions prevent the elicited responses from being effective at dealing with the source of stress (e.g. impeded escape in captivity). In a similar manner, developing nestlings of non-precocial species might be expected to be particularly vulnerable to the detrimental effects of chronic CORT elevations, since their nest-bound condition (precluding escape) and lack of necessary behavioral and physiological abilities to cope with ecological challenges (e.g. locomotion, flight, thermoregulation) could make them highly prone to suffering severe and long-lasting energetic imbalances. On the other hand, life-history theory predicts that animals should only display adrenocortical responses if these have the potential to exert fitness benefits on the individual, giving rise to an alternative scenario in which nestlings would be expected to shut-down HPA function until acquiring the capacity to perform proper adult-like responses against perturbations (i.e. around fledging age).

Such contrasting hypotheses have stimulated a growing number of studies investigating adrenocortical physiology during early life in a range of avian species (Sims and Holberton 2000; Love et al. 2003; Blas et al. 2005; Quillfeldt et al. 2009), in an attempt to uncover the actual mechanisms through which non-precocial nestlings trade-off the costs and benefits of CORT secretion throughout post-hatching development. According to the Developmental Hypothesis, this could be achieved by synchronising the maturation of the HPA axis (i.e. the capacity to secrete CORT) to the progressive development and acquisition of physiological, physical and behavioral abilities by the nestling as they approach independence.

The hypothesis was initially postulated by Sims and Holberton (2000), who demonstrated that young northern mockingbirds displayed attenuated HPA function as well as age-related increases in stress responsiveness throughout development. Since then, numerous empirical studies have reported similar patterns in several taxa, affording considerable credit to the theory (reviewed in Blas and Baos 2008). However, the precise proximate mechanisms behind these changes remain far from being fully understood. So far, experiments with northern mockingbirds (Sims and Holberton 2000) and Magellanic penguins (Walker et al. 2005) have demonstrated that nestlings injected with adrenocorticotropin hormone (which stimulates the adrenal glands) were capable of secreting CORT at levels comparable to those of adults, suggesting that their reduced ability to respond to stress during early development was not the consequence of an incomplete maturation of the adrenocortical tissue. Still, certain questions remain to be addressed, such as whether the regulation of ontogenetic patterns in CORT secretion occur at the hypothalamic or pituitary levels, whether it involves a maturation process or a deliberate down-modulation of endocrine activity (see Sapolsky and Meaney 1986 for a review in rats), and whether these patterns and potential mechanisms are shared across non-precocial avian taxa.

In any case, early nestling HPA hyporesponsiveness and lack of independent survival skills are typically offset by parental behaviors aimed at minimising the effect of adverse environmental conditions on their offspring (e.g. by providing food regularly, shielding from direct sun rays and rain; fending off predators; maintaining body temperature during cold weather; etc). A remarkable exception, however, is seen in certain avian species (e.g. many raptors and marine birds), where even though nestlings tend to display highly aggressive behaviors towards their siblings that often result in either facultative or obligate siblicide,

parents are only rarely observed to interfere or halt such disputes (Viñuela 1999; Ferree et al. 2004; Brewer et al. 2010). Therefore, nestlings of these species might be expected to benefit from displaying a fully functional HPA axis, since robust CORT elevations could allow them to deal adequately with such inescapable challenges at the nest scale (e.g. by promoting submissive behavior; Leshner et al. 1980).

In light of the above, we set out to determine whether adrenocortical responses in a facultatively siblicidal raptor (the black kite, *Milvus migrans*) followed the ontogenetic pattern of HPA axis maturation predicted by the Developmental Hypothesis. We also aimed to examine whether nestling plasma CORT levels would reflect the effects of a range of socio-ecological factors expected to raise allostatic load in this and other avian species. In particular, we predicted higher baseline and stress-induced plasma CORT levels in nestlings: 1) born in years of low inundation, since black kites heavily feed upon aquatic prey whose abundance is tightly linked to water levels (Bildstein et al. 1990; Cézilly et al. 1995; Sergio et al. 2011a); 2) sampled later in the season, because resource abundance declines as water bodies dry up and most late-breeders are young, low quality individuals (Sergio et al. 2009); 3) raised in poorer quality territories, where food availability is lower and parents are typically inexperienced (Sergio et al. 2009); 4) born in multiple-chick broods and lower down the brood hierarchy, because aggression and reduced access to food are likely to impose higher energetic costs (Hiraldo et al. 1990; Viñuela 1999; Viñuela 2000); 5) exposed to extreme ambient temperatures, since these induce the activation of expensive thermoregulatory responses (Thouzeau et al. 1999; Greño et al. 2008); 6) sampled in the early morning or late afternoon, because daily rhythms in CORT secretion have often been shown to peak right before the start of the active period and then gradually decline, to begin rising again by the start of inactivity (El-Halawani et al. 1973; Breuner et al. 1999;

Carere et al. 2003); 7) in poorer body condition, because CORT promotes maintenance through the mobilization of energy reserves and intensifies begging, which should encourage parents to increase provisioning rates (Kitaysky et al. 2001; Kitaysky et al. 2003); and 8) of masculine sex, given that their lower mass compared to females could make them less capable of coping with increased allostatic loads (Fargallo et al. 2003; Sergio et al. 2007). To test all these predictions, we analysed inter-individual variation in baseline and stress-induced plasma CORT levels in a sample of 115 black kite nestlings subjected to standardized capture and restraint series in Doñana National Park.

MATERIALS AND METHODS

Study area and species

Field work was conducted within Doñana National Park, which holds one of the largest breeding populations of black kites in the Iberian Peninsula (ca. 500 pairs; Madroño et al. 2004). The Park is located on the right bank of the Guadalquivir river estuary, covering 50,000 ha of marshland, sand dunes, Mediterranean scrublands and pinewoods between the provinces of Huelva, Seville and Cádiz (SW Spain). The Guadalquivir marshes typically flood in October-February as a result of autumn-winter rains, and progressively dry up with increasing spring and summer temperatures (García and Marín 2006). Therefore, depending on the annual climate regime, both the extent of the marshes and their hydroperiod can vary drastically from one year to the next.

Our model species, the black kite, is a medium-sized raptor with slight sexual dimorphism, females weighing on average 15% more than males (Sergio et

al. 2007). Populations of this species are often concentrated near large wetlands (Veiga and Hiraldo 1990; Sergio 2003; Tanferna et al. 2013), where breeding pairs establish territories of approximately 100m radius centered around their nesting tree (Viñuela et al. 1994; Sergio et al. 2011b). Egg laying is highly asynchronous at the population level, different pairs laying between late March and late May. Clutch size ranges between 1-3 eggs (very exceptionally four), laid at 2-3 day intervals (Viñuela and Carrascal 1999). At hatching, nestlings are often aggressive towards their siblings and exhibit frequent fights, especially during feeding bouts. Such behavior is thought to facilitate the establishment of a brood hierarchy where the oldest, first-hatched chick typically obtains the dominant position by winning most fights over food morsels on account of its larger size. However, as the hierarchy is settled and nestlings assume their respective rank positions (at around the third week of age), fighting gradually ceases (Viñuela 1999).

Field procedures

Between the months of June-July of 2008-2011 (average julian day = 105.4 ±1.2 SE; range = 86-137), we performed standardized capture and restraint stress series (Wingfield et al. 1997; Blas 2015) on 115 nestlings aged 16 to 48 days old (mean = 31.5 ± 0.7 SE) from a total of 75 black kite broods. Despite the well-established fact that physical capture and restraint induces an acute rise in plasma CORT levels after 3-4 minutes in birds (Romero and Romero 2002; Wada et al. 2007), we were uncertain whether our arrival and mere presence at the foot of a nesting tree would induce the same response in nestlings. Thus, to be conservative, we decided to sample only nests for which the configuration of the tree allowed a quick use of a ladder or rapid free climbing to reach the nestlings and collect the

first blood sample (T0) within a maximum of five minutes (mean = 2.87 ± 0.11 SE) from arrival at the foot of the tree, and a maximum of four (mean = 1.78 ± 0.08 SE) since their first handling. At the same time, we explicitly avoided over-sampling unusually low nests so as to minimize the risk of over-selecting individuals occupying low quality trees. The field team consisted always of a professional, fast tree-climber and at least three field workers on the ground to allow for the simultaneous bleeding of all nestlings in multiple-chick broods. Rank within the brood was assigned on the basis of visual inspection of differences in size and plumage development among siblings, which were always obvious and produced no discrepancies in opinion among observers. Next, nestlings were placed on the ground in a shaded area with their heads covered, leaving them undisturbed until subsequent blood-sampling at ten (T10) and at thirty (T30) minutes post capture (T10 mean = 11.29 ± 0.13 SE; T30 mean = 31.26 ± 0.15 SE). Immediately after collection, each blood sample (0.5ml drawn from the brachial vein) was transferred to a heparinized tube and stored in ice until arrival at the laboratory 2-4 hours later, when blood plasma was separated from the cellular portion by centrifuging for 10 minutes at 1430g. The resultant plasma supernatant was collected in empty screw-cap microtubes and stored, along with the cellular portion, at -80^{0}C for subsequent determination of CORT levels and molecular sexing analyses (Ellegren 1996) respectively.

Throughout our sampling activities, we kept records of the time of day (range = 7:00-21:00h) in order to account for the potential effect of diel fluctuations in plasma CORT levels. Finally, before returning the birds to their nests, nestlings were weighed (to the nearest 1g) and measured for tarsus, tail and 8th primary length (to the nearest 0.1, 1 and 1 mm respectively). Age of nestlings whose hatching had not been directly observed in earlier visits to the nest was estimated

using a logistic regression of 8th primary length on age (Hiraldo et al. 1990). Records of the maximum temperatures (mean = 30.6 ± 0.5 SE; range = 21.0 - 38.0 °C) and minimum temperatures (mean 14.6 ± 0.3 SE; range = 10.0 -20.0 °C) reached on the 24 hour period before sampling were obtained from the meteorological station of the Doñana Biological Reserve. Birds were handled and sampled following protocols approved by the Bio-Ethics Sub-Committee of the CSIC (references EBD11/09 and EBD11/25), in strict accordance with the national and European legislations concerning the use of animals for scientific purposes.

Plasma CORT assays

Plasma was extracted using an organic diethyl ether-based technique detailed elsewhere (Wayland et al. 2002). Extraction efficiency was calculated using three plasma samples spiked with a small amount (~5000 CPM) of [^{3}H] CORT. Samples were extracted in two batches and greater than 95% of the radioactivity was recoverable. Measurements were performed on extracts of the plasma samples reconstituted with 300µl of phosphate buffer saline (PBS; 0.05M, pH 7.6) and frozen at –20°C until CORT was measured by radioimmunoassay (RIA) as in previous studies (Blas et al. 2005). Antiserum and purified CORT for standards were purchased from Sigma Chemicals and [^{3}H] CORT from Amersham Bioscience. Each sample was measured in duplicate within the RIA. Serial dilutions of plasma extracts were parallel to the standard curve. All samples were at or above the limit of detection (ED80; mean ± SD = 12.66 ± 1.48pg/100µl). Samples were measured in seven separate assays, with average intra- and inter-assay coefficients of variation of 4.5% and 9.2%, respectively.

Statistical analyses

The level of inundation in each of the years of study (2008-2011) was estimated as the extent of flooded marshland during the sampling period (June-July). For this purpose, we accessed the available cloud-free Landsat MSS, TM and ETM+ images for similar dates and compared inundation masks between years. On the basis of these comparisons, we built an "inundation index" where 2010 and 2011 were categorised as "wet" years, 2008 as "intermediate" and 2009 as "dry". Territory quality was estimated through a Principal Components Analysis considering the percentage of years in which a territory had been occupied during the period 1989-2011, and the percentage area of marshland habitat within 2km of the nest, following previous work on this population (e.g. Sergio et al. 2009; Sergio et al. 2011b). Briefly, we loaded a georeferenced map of Doñana onto ArcGis software and plotted 2km-radius circles around each nest location using the ArcGis "buffer" tool. Next, we added a second layer of land-uses and used the "intersect" function to extract the area covered by marshland vegetation within each buffer, which we then calculated as a percentage of the total buffer area. ArcGis maps (shape files) were facilitated by the LAST-EBD group (http://last-ebd.blogspot.com). Average territory quality in our sample was 0.91 ± 0.08 SE, ranging between -3.29 and 1.71. To calculate body condition, we obtained the residuals of the quadratic regression of body mass on age, and expressed them as a proportion of the expected mass. The resulting body condition scores ranged between -0.39 and 0.33 (mean = 0.0001 ± 0.013 SE).

Bivariate correlations indicated that T0 CORT levels were unrelated to the time elapsed between arrival at the foot of the tree and blood sampling, or between capture and blood sampling ($r = 0.17$, $P = 0.10$; $r = 0.17$, $P = 0.11$ respectively),

and could therefore be safely considered to reflect baseline values (mean = 4.61 ± 0.30 SE, range = 0.92-13.69ng/ml). Maximum adrenocortical response to handling and restraint was taken as the highest CORT value obtained during the 30 minutes of restraint (regardless of whether this occurred at T10 or at T30 (Breuner et al. 1999); mean = 38.44 ± 1.53 SE, range = 6.80-80.72ng/ml). Four nestlings for which we lacked either one of the two acute measures were excluded from subsequent analyses.

Next, we built multivariate Generalised Linear Mixed Models (GLMM; Zuur et al. 2009) using Gaussian error structure and identity link function to examine the potential effect of each variable on plasma CORT while controlling for the influence of all other variables. Baseline (T0) and maximum stress-induced (T10 or T30) CORT levels were analysed separately. T0 CORT data were log-transformed in all cases to achieve normality. Saturated models included the following predictor variables: 1) factors: sex, inundation index (dry, intermediate, wet) and brood order (singleton, senior, 2^{nd}- or 3^{rd}-born chicks); 2) covariates: julian date, territory quality, body condition, time of day (hourly), time of day^2, maximum and minimum ambient temperatures in the 24 hours prior to sampling, age (days post-hatching) and age^2. The decision to include the variable "age" in its quadratic form was based on the presumption that ontogenetic increases in adrenocortical function might not necessarily follow a linear trend, but could instead stay low for most of development and increase sharply around fledging age (e.g. Quillfeldt et al 2007). Likewise, time of day^2 was considered in the saturated model to account for the possibility that diel rhythms in CORT secretion in nestlings resembled the pattern of early morning and late afternoon peaks exhibited by adults of several diurnal bird species (e.g. El-Halawani et al. 1973; Breuner et al. 1999; Carere et al. 2003). In addition, nest identity was fitted as a random term to control for the

potential pseudoreplication generated by multiple chicks sharing the same nest. Due to restrictions imposed by sample size, we intentionally avoided fitting interactions in order not to over-parameterize the model. The presence of potential collinearity issues between variables was discarded by checking Variation Inflation Factors prior to running the analyses (all VIFs < 4; Zuur et al. 2007). Model selection was conducted following a backward stepwise approach with significance level set at 0.05. The validity of the final models was assessed by inspection of the distribution of the residuals. Statistical analyses were performed using R 3.0.3 (R Development Core Team 2009) and IBM SPSS Statistics 22.0.0.

RESULTS

Baseline CORT

The best final model retained only the effects of age (χ^2 = 3.78, df = 1, P = 0.05) and time of day^2 (χ^2 = 6.39, df = 1, P = 0.01). The relationship between baseline CORT and age was linear and positive, meaning that hormone levels increased progressively across the nestling period (Fig. 1A). Baseline levels were also higher in nestlings sampled early in the morning, declined gradually to reach minimum levels during the central part of the day, and increased again in the afternoon (Fig. 2A). Interestingly, nestlings born in years of drought exhibited a marked tendency to display higher plasma CORT levels than nestlings born in years of either intermediate or high marshland flood levels, although the difference did not receive statistical support (Fig. 3A).

Maximum stress-induced CORT levels

The best final model revealed that maximum stress-induced CORT levels varied with age (χ^2 = 11.12, df = 1, P < 0.01), time of day[2] (χ^2 = 7.86, df = 1, P < 0.01) and with the yearly inundation index of the marshland (χ^2 = 5.84, df = 1, P = 0.05). Specifically, maximum acute CORT levels augmented with nestling age (Fig. 1B) and were higher in nestlings sampled just after sunrise and before sunset than in those sampled during the central hours of the day (Fig. 2B). Finally, nestlings sampled on a dry year exhibited the most robust CORT responses to capture and restraint, followed by nestlings sampled on years of high and, least of all, intermediate flood levels (Fig.3B).

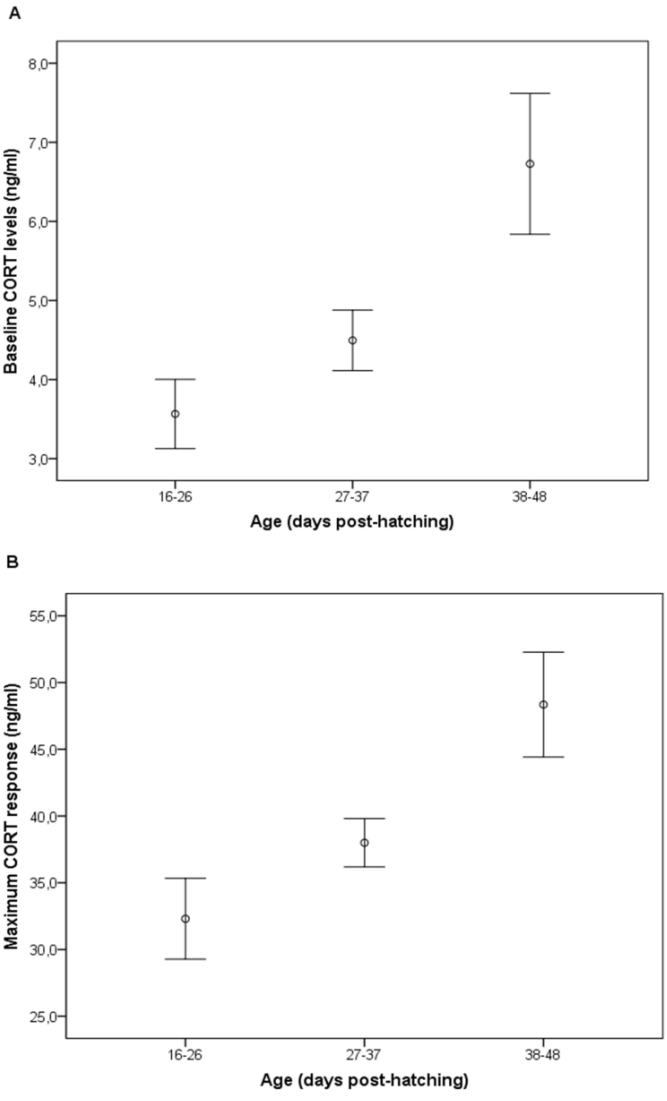

Fig. 1: Age-related changes in nestling plasma CORT levels. Ontogenetic variation in (A) baseline CORT levels and (B) maximum CORT response following capture and restraint in nestling black kites. Bars represent ± 1 SE.

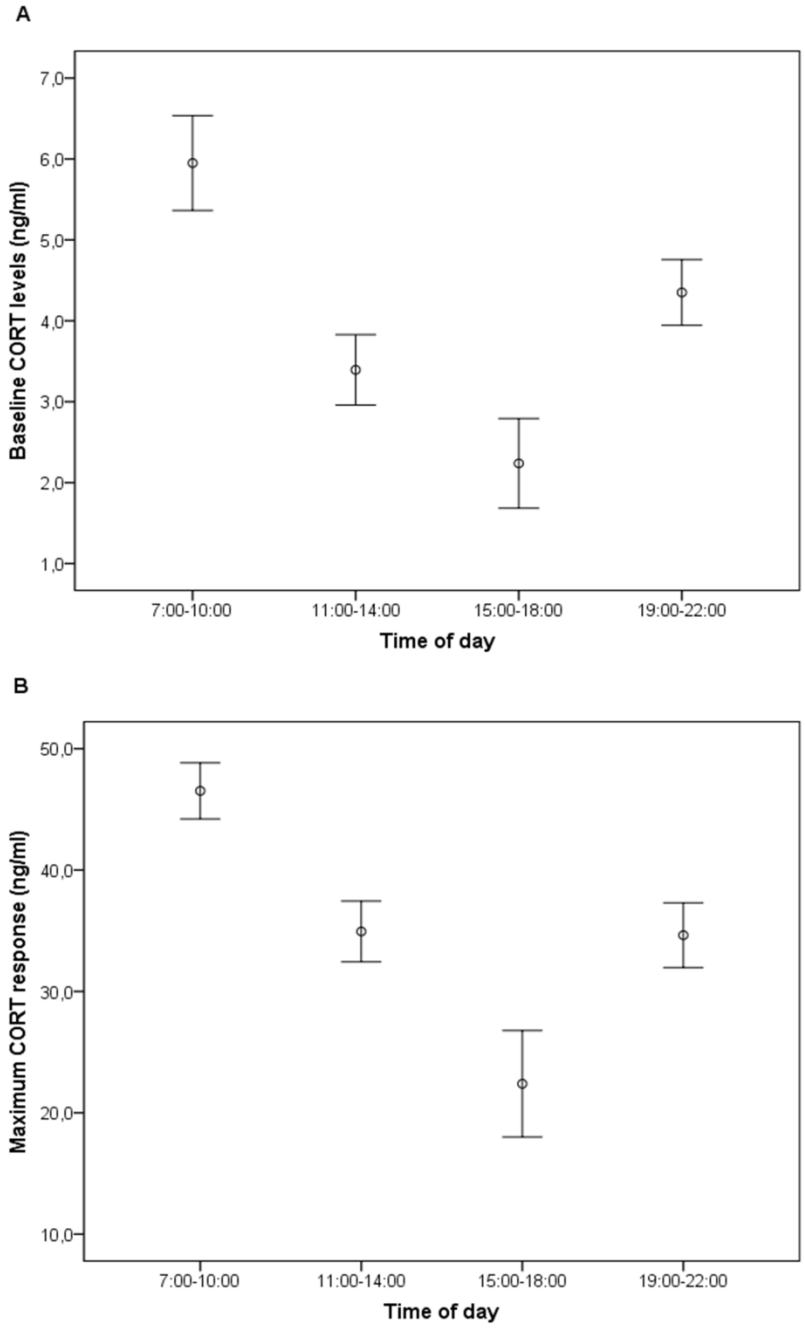

Fig. 2: Diel fluctuations in nestling plasma CORT levels. Variation in (A) baseline and (B) maximum stress-induced CORT levels of black kite nestlings in relation to time of day. Bars represent ± 1 SE.

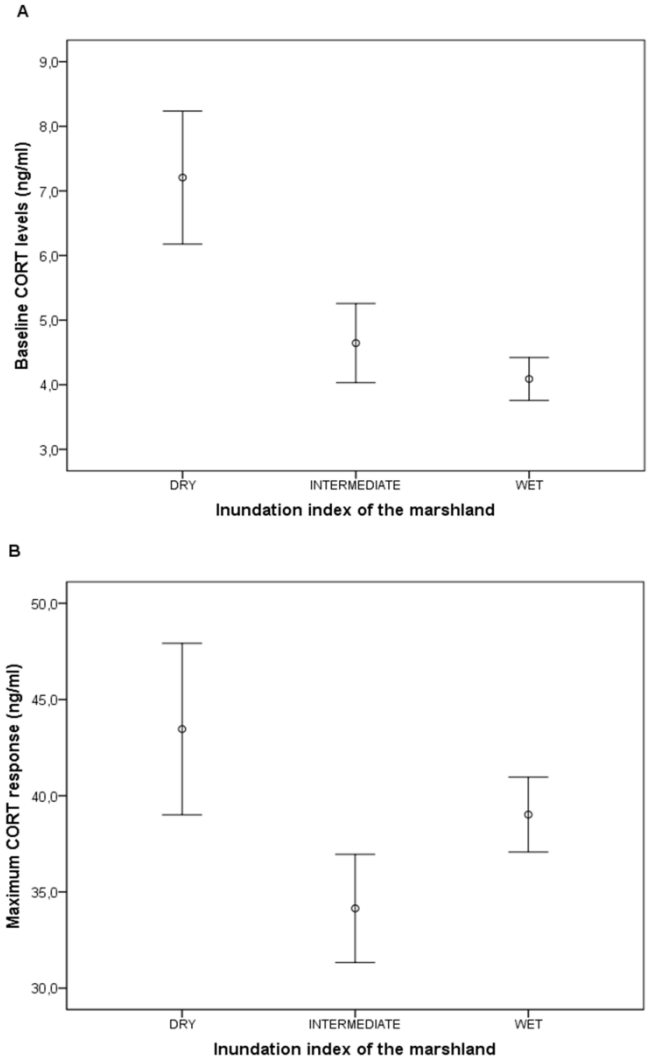

Fig. 3: Variation in nestling plasma CORT levels in relation to the inundation index of the marshland. Mean values of (A) baseline and (B) maximum stress-induced CORT levels in nestling black kites sampled in Doñana National Park on years of low (dry), intermediate and high (wet) flooding of the marshlands. Bars represent ±1 SE.

DISCUSSION

Average baseline and stress-induced plasma CORT levels in black kite nestlings were below the mean values reported for conspecific adults (Blas et al 2011) but well within the ranges previously reported for chicks of this species (Blas 2015) and many other non-precocial species during development (Blas and Baos 2008 and references therein). Furthermore, consistent with the Developmental

Hypothesis (Sims and Holberton 2000; Blas and Baos 2008), our results revealed an age-related increase in adrenocortical responsiveness that likely reflects a gradual, ontogenetic maturation/upregulation of HPA function. In adult birds and precocial chicks, robust increases in circulating CORT in response to noxious stimuli are expected to confer fitness advantages by inducing a number of changes in behavior and physiology aimed at lowering heightened allostatic loads back to energetically affordable levels (Holmes et al. 1990; McEwen and Wingfield 2003; Jenkins and Porter 2004; Blas 2015). However, non-precocial nestlings lack the ability to carry out many of the adjustments necessary to escape or overcome challenges, especially during the first weeks after hatching, when chicks are practically unable to locomote, thermoregulate or defend themselves from predators, weather inclemencies, parasites or disease. On account of such limitations, the capacity to activate a complete adrenocortical response would not only lack the expected fitness value, but could, on the contrary, be potentially damaging to nestlings given that sustained elevations of plasma CORT during days or weeks can exert deleterious effects on development, including retarded growth, reduced cognitive abilities, weakened feathers and compromised immune function (Kitaysky et al. 2003; Elftman et al. 2007; DesRochers et al. 2009). At the other extreme, complete suppression of adrenocortical function could also prove detrimental if nestlings are already capable of displaying some physiological and/or behavioral adjustments to cope with the perturbation. In agreement with the latter, we found that all sampled nestlings experienced plasma CORT elevations in response to capture and restraint, thus suggesting that some degree of responsiveness is necessary even during development to facilitate regulation of homeostatic processes. For example, in facultatively siblicidal species like the black kite, CORT elevations may promote the

expression of aggressive and submissive behaviors that can be clearly adaptive in the context of social challenge (Leshner et al. 1980; Blas et al. 2011).

Interestingly, while the mounting evidence for ontogenetic improvements in the adrenocortical response to stress (i.e. acute CORT elevations) seems consistent across non-precocial birds (e.g. Sims and Holberton 2000; Blas et al. 2006; Wada et al. 2007; Müller et al. 2010), it remains less clear whether the same should hold true for baseline CORT levels. Overall, age-related elevations in baseline CORT titers have been documented in some non-precocial species (Schwabl 1999; Love et al. 2003), while other studies have reported no age effects (Sims and Holberton 2000; Blas et al. 2006; Wada et al. 2007; Quillfeldt et al. 2009) or even the opposite pattern (i.e. ontogenetic decreases; Walker et al. 2005; Romero et al. 2006; Dehnhard et al. 2011). In this study, black kite nestlings exhibited a gradual rise in baseline CORT levels across all sampled ages. Non-precocial chicks are born totally or partially blind, weak and relatively immobile, but rapidly become increasingly active, gaining strength, coordination and mobility as they start building body muscle. In this sense, the reported age-related elevations in baseline CORT could be serving to promote the mobilisation of glucose and its conversion from lipid stores to deliver the necessary energy to fuel the increasingly heightened activity levels of growing chicks (Sapolsky et al. 2000). At the same time, larger nestlings require more food, which must be obtained from their parents. Given that raised CORT concentrations have been shown to encourage begging behavior in several other bird species (Kitaysky et al. 2001; Kitaysky et al. 2003), ontogenetic increases in baseline CORT in black kites could therefore also be reflecting the physiological mechanism by which nestlings urge parents to intensify provisioning efforts in order to meet their growing metabolic demands throughout development. In support of this view, peak concentrations were reached around fledging age. Interestingly,

heightened HPA activity at this precise stage seems to be a particularly common trait in many non-precocial birds, even among species that do not exhibit similar increases in baseline CORT levels at younger ages (Sims and Holberton 2000; Kern et al. 2001; Quillfeldt et al. 2007; Blas and Baos 2008). The reason for this seems to be related to the sudden rise in the allostatic load that these nestlings experience as they approach nest abandonment (e.g. associated with flight activities, reduced feeding rates from parents to stimulate fledging, etc.), which is likely more pronounced at this stage than across earlier developmental stages. Likewise, significant baseline CORT elevations have also been reported to take place at various other highly demanding life-history transitions across vertebrate classes, such as parturition, metamorphosis, or juvenile dispersal (Wada 2008), thus highlighting the important role of CORT as a mediator of essential physiological processes for maintaining energy balance. As further proof, nestlings of non-precocial but ground-nesting species, which typically postpone their first flights until days or weeks after nest abandonment, and are therefore unlikely to experience similarly heightened energetic demands at this stage, have been shown to lack equivalent increases in baseline CORT (Romero et al. 2006).

Overall, the reported rhythm in daily adrenocortical function, with reduced CORT titers in the middle of the day, closely resembled those previously described in adult and juvenile individuals of several diurnal passerine (Breuner et al. 1999; Romero and Remage-Healey 2000; Rich and Romero 2001; Carere et al. 2003) and non-passerine bird species (El-Halawani 1973; Joseph and Meier 1973). Such widespread pattern suggests that the environmental cues regulating diel rhythms in CORT levels could be consistent across species and life stages. In this respect, both experimental and field studies have repeatedly pointed to the potential role of photoperiod on diel HPA function, since hormone secretion generally tends to peak

just before or after dawn, declines progressively through the day, and rises again just before or after dusk. However, contrary to a photoperiod-related diel rhythm hypothesis, nocturnal bird species have been shown to exhibit the reverse pattern, with higher CORT levels during the day and lower at night (Dufty and Belthoff 1997; Roulin et al. 2010). These contrasting results have nourished a growing appreciation that daily CORT fluctuations may not respond to the stimulus of light-dark cycles *per se*, but rather to the alternation of periods of activity and inactivity. Under this scenario, the characteristic "pre-activity" peak in adrenocortical function has been posited to serve to induce the mobilisation of glucose and fat stores that would help bring the animal into an appropriate physiological state to meet the energetic demands associated with the start of the active period (e.g. begging, feeding, fighting among siblings, etc.), regardless of the species-specific diurnal or nocturnal activity mode. While we do not currently hold data on nestling daily activity patterns, which would clearly be useful to reinforce this argument, prey delivery rates in other birds have often been reported to peak during the first morning hours, in the case of diurnal bird species or, in the case of nocturnal birds, at the beginning of the night. Furthermore, some of these species have even been shown to exhibit a second feeding peak before the end of their respective day or night active periods, offering remarkable examples of parallelism between avian feeding activity patterns and diel rhythms in CORT secretion which support a potential causal link (Morton 1967; Holthuijzen 1990; Delaney et al. 1999; Margalida and Bertran 2000; Zárybnická 2009; Beaulieu and Schaefer 2014).

The inundation index of the marsh was another significant predictor of maximum stress-induced nestling CORT levels. More specifically, nestlings born on the driest year reached the highest CORT values in response to handling and restraint, followed by nestlings born on years of high, and last of all, intermediate

flood levels. Likewise, drought conditions were found to be similarly, though non-significantly, associated with a marked elevation in nestling baseline CORT concentrations. Large aquatic ecosystems represent an important source of prey for our model species. It is not surprising, therefore, that the availability of this habitat type constitutes a major driver of settlement and foraging patterns in black kites, as evidenced by data collected during intensive surveys and radio-tracking studies on this and other populations (Sergio et al. 2003; Sergio et al. 2011a; Tanferna et al. 2013). However, in temporarily inundated environments, such as the Doñana marshes, profitability tends to be highly variable and subject to the dynamics of flooding-desiccation cycles. The reason for this lies in the fact that the abundance of aquatic prey is tightly linked to water levels, which vary depending both on the frequency and intensity of winter rainfalls, and on spring-summer temperatures (García and Marín 2006; Sergio et al. 2011a). The effects of such association are especially evident in years of drought, when the marshes never flood at all in the winter and the availability of aquatic prey is drastically reduced, resulting in an overall decline in habitat quality for black kites. Among adults, for instance, performance in a number of reproductive parameters, including hatching rates, brood size and fledging success has been shown to be significantly depressed under these conditions (Sergio et al. 2011a). In the same context, we believe that the considerably heightened response to stress of nestlings born on the driest of the four study years could possibly be reflecting a situation of heightened allostatic loads induced by reduced parental provisioning rates and poorer overall body condition. On top of this, indirect effects of low prey availability could further contribute to raising CORT levels, for example, by promoting an escalation in the frequency and intensity of aggressions among siblings during feeding bouts (Viñuela 1999). Alternatively, females could be forced to leave their nests unguarded for long

periods of time to help their mates in procuring sufficient food to raise the brood, thus potentially rendering nestlings vulnerable to exposure to inclement environmental conditions (e.g. direct sun exposure) or predation attempts (e.g. through an increase in the number of intra- and inter-specific territorial intrusions). Following this line of thought, it would be interesting to explore whether the environmental context in which nestlings develop affects the strength or direction of the relationship between CORT levels and other socio-ecological factors. Unfortunately, the sample size in this study did not allow us to conduct such analysis.

In turn, the reverse argument (i.e. that high prey delivery rates lower the allostatic load of nestlings) could likewise explain why black kite nestlings raised in a year of intermediate flooding were observed to display lower adrenocortical responses to handling and restraint than nestlings raised in years of high flooding, since shallow water (down to a certain limit), may favour the concentration of aquatic prey (e.g. fish and crayfish), and/or make them more easy to detect and catch by predators (Bancroft et al. 2002; Chiu et al. 2013).

Contrary to our predictions, we could not detect the effect of any of the remaining socio-ecological variables that we tested (i.e. territory quality, sampling date, order within the brood hierarchy, sex, body condition and ambient temperature) on plasma CORT levels. This lack of positive results may suggest that parents have efficiently dampened the impact of environmental perturbations on nestling physiology through the modulation of parental behaviors such as brooding or food provisioning. However, it must be noted that very young black kite nestlings were generally difficult to bleed, especially within the time frame of 4 minutes from capture. This unforeseen handicap forced us to restrict the study to

nestlings in their middle-late stages of development (range = 16-48 days), when the bulk of mortality attributable to brood reduction is expected to have taken place already (Viñuela 1999). In this sense, the lack of effect of other ecological variables on adrenocortical function could be explained by the fact that we may have sampled the best quality portion of the nestling population, i.e. individuals that had survived an important natural bottleneck and were likely in good enough condition to cope successfully with environmental challenges and their associated energetic demands. On similar grounds, the absence of significant differences in CORT levels between nestlings of different hatching order could be the consequence of having excluded the ages of peak hostility among siblings, as it is mainly during the first 2-3 weeks post-hatching when nestlings fight most to establish their respective positions within the brood hierarchy (Viñuela 1999).

To circumvent this problem, future studies could contemplate the use of alternative or complementary, less invasive sampling techniques, such as the analysis of CORT levels in fecal samples (Lobato et al. 2008) or feathers (Bortolotti et al. 2008). These may partially remove some of the technical difficulties associated with bleeding very young birds and may better reflect challenges faced over longer time-periods. We believe that the integration of complementary techniques could yield promising results, especially for the management of species declines, often fuelled by different threats operating at different scales and thus requiring more robust and comprehensive knowledge for conservation planning.

In summary, both baseline plasma CORT levels and adrenocortical responsiveness in black kite nestlings increased with post-hatching age, suggesting that HPA maturation/upregulation likely paralleled the progressive acquisition of

physiological, physical and behavioral abilities associated with ontogenetic development. Likewise, diel rhythms in HPA function were also apparent in both baseline and acute CORT titers. So far, the few previous studies that have attempted to characterize daily CORT fluctuations in developing birds have been conducted either on chicks of precocial and diurnal (e.g. Lauber et al. 1987) or non-precocial but nocturnal avian species (Quillfeldt 2007; Roulin et al. 2010). Thus, our results using nestlings of a non-precocial, diurnal avian species as the study model broaden the current picture of daily variation in HPA function in birds. One of the reasons for such previous gap of knowledge lies in the fact that most previous studies of this sort have controlled for the potential effect of diel rhythms in CORT secretion by establishing restricted time frames for the collection of blood samples, unknowingly of whether this rhythmicity was in fact taking place or whether it even resembled that of adults. Here, by sampling nestlings at different times during the day instead of at fixed hours, we provide empirical evidence that daily patterns in CORT levels can be robust already in 3-9 week old nestlings. Thus, although the question of whether diel rhythms arise during pre- or post-hatching development remains open to further research, our results confirm that these can exist from an early age, constituting a source of variation which must be accounted for in experimental designs. Finally, nestlings raised in a year of drought exhibited higher baseline CORT levels and responded more robustly to stress by reaching higher CORT concentrations than nestlings raised in years of intermediate or high inundation of the marshland. This finding might be particularly relevant in the current context of climate change, where shifts in rainfall regimes towards increasingly drier seasons (IPPC 2013) could lead to chronic CORT elevations and/or heightened adrenocortical responses in nestlings, potentially resulting in deleterious effects on development and long-term fitness (Blas et al. 2007).

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CAPÍTULO 2

Ambient temperature, body condition and sibling rivalry explain feather corticosterone levels in developing Black kites (*Milvus migrans*)

Ambient temperature, body condition and sibling rivalry explain feather corticosterone levels in developing Black kites (*Milvus migrans*)

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ABSTRACT

In birds, the steroid hormone corticosterone (CORT) is considered a major mediator in a number of physiological mechanisms and behaviours critical for the regulation of an organism´s energy balance. However, the question of whether the way in which circulating plasma CORT is incorporated into feathers actually reflects this regulation, and consequently whether feather CORT (CORT-F) measures allow to make inferences on past levels of energetic demands (i.e allostatic load), remains unclear. While previous studies have yielded promising results, the technique still wants further study and validation in a wider range of bird species, across life history stages and under different ecological situations. Here we tested whether CORT-F levels in a sample of 427 nestling Black kites (*Milvus migrans*) reflected inter-individual variation in a number of environmental, social and physiological variables known to elevate allostatic load during development. We found that nestlings exposed to low ambient temperatures exhibited high CORT-F, suggesting that feathers registered the expected rise in circulating CORT levels inducing thermogenesis in response to cold stress. CORT-F was also negatively related to body condition, supporting the notion that allostasis becomes less affordable as energy stores become depleted. Finally, CORT-F also reflected brood hierarchy rank, with subordinate chicks exhibiting the highest levels, followed by first-hatched (dominant) chicks and lastly singleton nestlings. Moreover, CORT-F levels in nestlings from multiple-chick broods (but not in singletons) declined with age, mirroring the dynamics of establishment and stabilization of sibling hierarchies in Black kites. Altogether, our results underscore

the potential of using CORT-F to infer allostatic load in wild avian populations and their potential power as indicator tools in conservation biology.

INTRODUCTION

In animals, the ability to maintain certain essential physiological systems within their optimal ranges (i.e. *homeostasis*) is critical for survival. Paradoxically, such systems' stability can only be attained through changes in a number of physiological mediators that allow organisms to respond and adjust to a constantly changing environment; a process known as *allostasis* (McEwen & Wingfield 2003). In birds, the hormone corticosterone (CORT: the end-product of the hypothalamic-pituitary-adrenal (HPA) axis and a hallmark of the response to stress) is considered a major allostasis mediator (reviewed in Blas 2015). Circulating CORT levels typically raise in response to energy demanding situations, and promote a suite of physiological and behavioural changes aimed at meeting such demands (also termed *allostatic load*) associated to both predictable (e.g. life-history stages, seasons, environmental rhythms; Landys, Ramenofsky & Wingfield 2006; Wada 2008) and unpredictable events (e.g. predation attempts, agonistic interactions; Leshner 1980; Cockrem & Silverin 2002). For example, elevated CORT levels can induce gluconeogenesis and the mobilisation of fat stores (to increase the availability of usable energy; Sapolsky, Romero & Munck 2000), or promote locomotor activity and foraging behaviour (to avoid predators and ensure feeding; Kytaysky et al. 2003; Crossin et al. 2012). Although short-term elevations of

circulating CORT are highly adaptive, sustained elevations (typically associated to chronic stress), have the potential to exert diverse deleterious effects in the long term (e.g. reducing growth, immune system suppression, cognitive abilities, etc.; Kitaysky et al. 2003; Elftman et al. 2007; Fairhurst et al. 2013).

Field ecologists have traditionally quantified circulating (plasma) CORT levels as a tool to monitor adrenocortical function in wild birds, allowing to infer exposure to energy demanding and stress-related situations in wild birds (Kitaysky, Wingfield & Piatt 1999; Newcomb Homan et al. 2003; Blas 2015). Only recently, laboratory techniques have been developed to quantify CORT levels in feather samples (CORT-F; "feather corticosterone"), offering a non-invasive tool for resolving the underlying physiological mechanisms that link environmental conditions to internal energy regulation processes and behaviours. The finding that newly growing feathers incorporate circulating molecules of CORT into their forming keratin structures (Bortolotti et al. 2008; Jenni-Eiermann et al. 2015), acting as records of past adrenocortical function, has significantly broadened the potential of field research by removing many of the methodological and ethical complications associated to blood sampling (e.g. observer interferences, bird capture and handling, sample preservation; see Blas 2015 for details). Besides, current efforts to identify external sources of CORT deposition, such as preen oils, have been sterile (Bortolotti et al. 2008; Lattin et al. 2011), thus reinforcing the assumption that CORT-F can reliably reflect the history of adrenocortical activity (i.e. fluctuations in baseline levels plus temporary acute elevations) experienced by an individual in response to allostatic demands during the very defined period encompassing feather growth. So far, a growing number of studies have successfully reported detectable CORT-F levels in a range of ecologically and phylogenetically distant bird species (e.g. Lattin et al. 2011; Carrete et al. 2013; Kouwenberg et al.

2013,) in which CORT-F concentrations have been associated to experimentally elevated and sustained levels of plasma CORT (Fairhurst et al. 2013), reproductive parameters (Bortolotti et al. 2008; Crossin et al. 2013; Kouwenberg et al. 2013), growth rates (Fairhurst et al. 2013), nestling survival (Fairhurst et al. 2013), nutritional state (Crossin et al. 2013; Legagneux et al. 2013; Will et al. 2014), travelling distance/home range (Carrete et al. 2013; Bourgeon et al. 2014), and environmental and seasonal factors (Bortolotti et al. 2008; Fairhurst et al. 2012; Legagneux et al. 2013). Despite this growing interest, however, our understanding of the information conveyed by variations in CORT-F levels is still in its infancy, and the need for further studies to help resolve important ecological questions is pressing.

Here, we test the hypothesis that CORT-F levels reflect inter-individual variation in the energetic demands experienced during development, using semi-altricial Black kite (*Milvus migrans* Boddaert) nestlings as a study model. In Black kites, juvenile (i.e. definitive) feathers start to emerge when nestlings reach approximately 12 days of age (Hiraldo, Veiga & Mañez 1990) and continue growing past fledging age (c.a. 45 days post-hatch, authors' data), providing a suitable time-frame to analyse the influence of a range of natal environmental conditions known to cause variability in allostatic loads (i.e. differences in the energetic requirements of chicks throughout post-hatching development). Using feather samples from 427 nestlings of different ages, nests and social environments collected during seven consecutive breeding seasons (years 2006-2012), we tested the prediction that higher CORT-F levels would be associated to: 1) nestlings reared in low-quality territories, because these have lower food resources, are owned by lower quality parents and may afford little protection to nestlings from predators or adverse weather (Sergio et al. 2009a); 2) nestlings exposed to inclement

temperatures as these challenge thermoregulation and homeostasis (Thouzeau, Duchamp & Handrich 1999; Greño, Belda & Barba 2008); 3) nestlings showing compromised body condition, because this generally reflects an imbalance between available versus required energy (Kitaysky et al. 2001a); 4) nestlings born late in the breeding season, because food resources become progressively scarcer, and younger, less experienced individuals usually lay later in the season (Sergio et al. 2007a; Sergio, Blas & Hiraldo 2009b; Sergio et al. 2011a); 5) older nestlings, because they will have a more developed HPA axis (Developmental Hypothesis; Sims & Holberton 2000; Blas et al. 2006; Blas & Baos 2008); 6) junior nestlings, because brood hierarchies are established by means of aggressive disputes among siblings, and last-hatched chicks are expected to be more vulnerable to the attacks from their older siblings on account of their smaller size and competitive inferiority (Hiraldo et al. 1990; Viñuela 1999; Viñuela 2000); and 7) male nestlings, because their lighter weight compared to females could make them more susceptible to decreased food intake and aggression from siblings (Fargallo et al. 2003).

MATERIALS AND METHODS

Study area and species

Our study was conducted in Doñana National Park (south-west Spain). The area is characterised by a highly heterogeneous landscape dominated by sand dunes, pinewoods, scrublands and seasonally flooded marshland. Mean daily temperatures during the Black kite's nestling months (May-July) are typically in the range of 13 to 31.5°C, with maximums that can reach up to 43°C.

The Black kite (Fig. 1) is a medium-sized semi-social raptor that breeds in

loose aggregations, often in close association with large bodies of water. Females are generally heavier than males (15% on average; Sergio et al. 2007b), and lay clutches of 1-4 eggs at 2-3 day intervals. Incubation lasts approximately 30 days and starts as soon as the first egg is laid (Viñuela & Carrascal 1999). As a result, hatching is typically asynchronous, allowing an important size advantage for the senior chick, which acquires dominant status through frequent aggressions towards its younger siblings (often resulting in serious injuries; Viñuela 1999). This behaviour, typical in all multiple-chick broods, is thought to have evolved as a mechanism to promote the establishment of stable hierarchies for priority access to food, which should serve to facilitate brood reduction in the event of food scarcity (Viñuela 1999; Sergio et al. 2011a). However, once the hierarchy is settled, aggressions gradually decline, to become practically absent during the late stages of the nestling period.

Fig. 1 Black kite. Photograph taken by Fabrizio Sergio.

Field procedures

Black kite territories were visited at the start of the breeding season to look for signs of reproductive activity (courtship displays, nest construction, copulations, territorial defence, etc.). Active nests were then visited at least three times: (1) during incubation, to record clutch size; (2) shortly after hatching, to determine brood size and nestling age, and (3) approximately ten days before the estimated fledging date to record the number of nestlings raised to fledging age (Sergio et al. 2014). Whenever possible, mass and morphometric measures (8^{th} primary, tail and tarsus length) were taken from all nestlings (mean age = 31.3 days, age range = 19-55 days). During the last nest check, nestlings were banded with metal and alpha-numeric coded plastic rings designed to facilitate post-fledging identification with the use of spotting scopes. Finally, two supra-tail covert feathers were removed from each bird and stored at -21^{0}C in individual plastic bags until subsequent analyses of CORT-F levels following Bortolotti et al. 2008 (see details below). All nestlings were sexed by molecular methods.

In Black kites, feather growth rates are remarkably constant during development, and feather length can thus be used to estimate nestling age (Viñuela & Bustamante 1992). We used a regression of 8^{th} primary length on age to backdate the birth date of nestlings whose hatching was not directly observed. Rank within the brood was assigned on the basis of visual inspection of differences in size and plumage development among siblings. Rank assignment was always obvious, with no discrepancies in opinion among observers. Body condition was calculated as the residuals of the quadratic regression of body mass on age.

In order to assess the effect of ambient temperatures on CORT-F levels, we calculated the average of the mean daily temperatures experienced by each nestling

during the period of feather growth. Since tail coverts emerge around day 12 (at the same time as the 8th primary; Hiraldo et al. 1990; Viñuela & Bustamante 1992; authors, pers obs) and are still growing when nestlings reach fledging age, we estimated feather growth periods (in days) for each individual chick as its age at the time of sampling minus 12. Temperature records for the calculated dates were taken from the meteorology station of the Doñana Biological Reserve.

Finally, territory quality was estimated through a Principal Components Analysis considering the percentage of years in which a territory was occupied and the percentage area of marshland within 2 km from the nest (Sergio & Newton 2003; Sergio et al. 2009a; Sergio et al. 2011a; Sergio et al. 2011b).

Feather CORT analysis

Extraction of CORT from feathers was carried out using a methanol-based technique (see Bortolotti et al. 2008 *Supplementary Appendix S1*). First, the length of each feather was measured, excluding the calamus which was removed. Then, each feather was cut into pieces of < 5 mm^2 and placed in a glass vial to which 10 ml of methanol was added (HPLC grade, VWR International, Mississauga, ON). The vials were placed in a sonicating water bath at room temperature for 30 min, followed by incubation at 50 °C overnight in a shaking water bath. Using vacuum filtration, the methanol containing the hormones was separated from the feathers, washing vials with additional 5ml of methanol and adding it to the original methanol extract. Vials with the methanol extract were placed open in a 50 °C water bath in a fume hood under air for evaporating until they were completely dry. When the evaporation of the samples was completed, the extract residues were reconstituted with 1200 µl of phosphate buffer (PBS; 0.05M, pH 7.6) and frozen at

–20 °C until CORT was measured by radioimmunoassay (RIA). Seven extraction processes were carried out in total. To determine the recovery of the extraction, three feather samples per batch were spiked with a small amount (~5000 CPM) of [^{3}H] corticosterone and the percentage of radioactivity recovered was assessed. Greater than 90% of the radioactivity was recoverable in the reconstituted samples. CORT concentration was determined using RIA, as described in Wayland et al. (2002) and Bortolotti et al. (2008). Measurements were performed on duplicate reconstituted extracts. Serial dilutions of feather extracts were parallel to the standard curve. Antiserum was purchased from Sigma Chemicals (product C8784, lot number 090M4752) and purified CORT for standards were purchased from Amersham Bioscience. Assay variability was determined as the % coefficient of variation (CV) resulting from repeated measurement of samples spiked with a known amount of CORT in each assay. We performed 12 assays with a mean intra-assay CV of 4.9 %, and inter-assay CV of 17.4 %. We expressed CORT-F values as pg cort mm^{-1} feather, since deposition of CORT into growing feathers is hypothesized to be time dependent, not mass dependent, and feather length is the most valid estimate of unit time of feather growth (see Bortolotti et al. 2008; Bortolotti et al. 2009; Bortolotti 2010; Jenni-Eiermann et al. 2015).

Statistical Analyses

The influence of natal environmental conditions on nestling CORT-F levels was examined by running Generalized Linear Mixed Models (GLMM, Zuur et al. 2009) with Gaussian error structure and an identity link function. Sex, brood order (four levels: single, first-, second- and third-hatched chicks) and year (seven levels: years 2006-2012) were included as categorical variables, while body condition,

territory quality, hatching date, age and ambient temperature were fitted as continuous predictor variables. Nest identity was included as a random term to control for the potential pseudoreplication generated by multiple chicks from the same nest. Models were constructed considering all the variables above. Only meaningful interactions according to our knowledge of the species' biology and ecology were tested, including brood order x sex; brood order x age; brood order x hatching date; brood order x body condition; body condition x sex; body condition x age; age x ambient temperature; territory quality x hatching date; year x body condition; year x hatching date; year x territory quality; year x ambient temperature; year x age. To avoid over-parameterization, each interaction was tested one at a time by removing it from the full, saturated model (i.e. the one with all main effects plus the interaction of interest). In all cases, CORT-F (response variable) was ln-transformed to achieve normality. Model selection was conducted following a backward stepwise approach with significance level set at 0.05. The validity of the final models was assessed by inspection of the distribution of the residuals, which complied in all cases with the assumptions of normality and homogeneity of variance. All statistical analyses were performed using R 3.0.3 (R Development Core Team 2009).

RESULTS

In the final model, CORT-F increased with both decreasing ambient temperature (estimate ± SE = -0.038 ± 0.01; likelihood ratio test: $\chi^2 = 13.38$, df = 1, $P < 0.001$, Fig. 2), and deteriorating body condition (estimate ± SE = -0.26 ± 0.11;

likelihood ratio test: χ^2 = 5.07, df = 1, P = 0.02). The interaction between age and brood order was also significant (estimate ± SE = -0.26 ± 0.11; likelihood ratio test: χ^2 = 9.91, df = 3, P = 0.02). CORT-F in singleton nestlings remained constant throughout development, while it declined gradually with increasing age in nestlings from multiple broods (Fig. 3). The rates of decline, however, were steeper for second- and third-hatched nestlings (slopes = -0.49 and -0.37 respectively) than for first-hatched nestlings (slope = -0.16). As a result, differences in CORT-F levels between single, senior and junior nestlings were maximal during the early stages of development, but disappeared at approximately 35-40 days of age. When the effect of brood order was tested separately in nestlings younger and older than 35 days, differences between nestling categories were only found in the former group (Tukey´s HSD post-hoc test; Fig. 4). Thus, during early development, singletons deposited significantly less CORT-F than any of the nestlings born in multiple-chick broods (in all cases P < 0.05), while senior nestlings from multiple-chick broods in turn deposited less CORT than second-hatched nestlings (P < 0.01). Third-hatched nestlings exhibited similar CORT-F levels to second-hatched nestlings (P = 0.99), however, they were also statistically undistinguishable from those of senior nestlings (P = 0.34), probably as a consequence of small sample size affording reduced statistical power in this last group (Fig. 4).

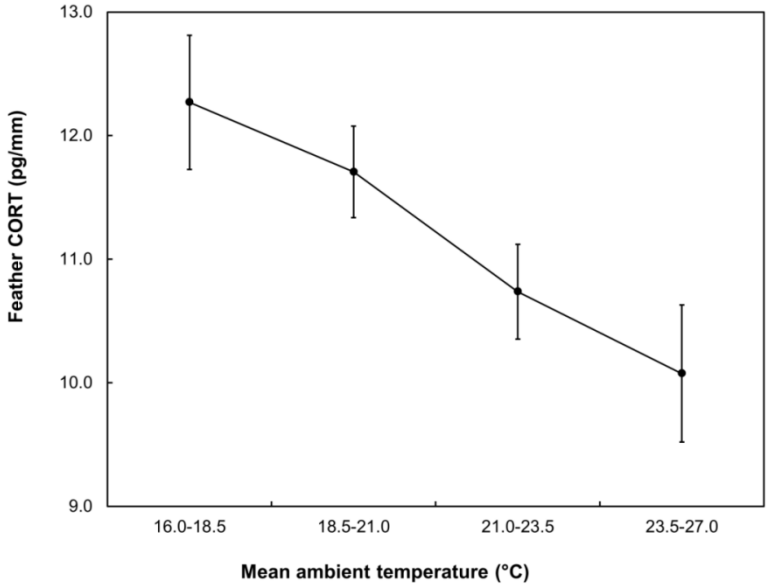

Fig. 2. Relationship between feather corticosterone levels and mean ambient temperature experienced by nestlings during the days in which they were growing their feathers.

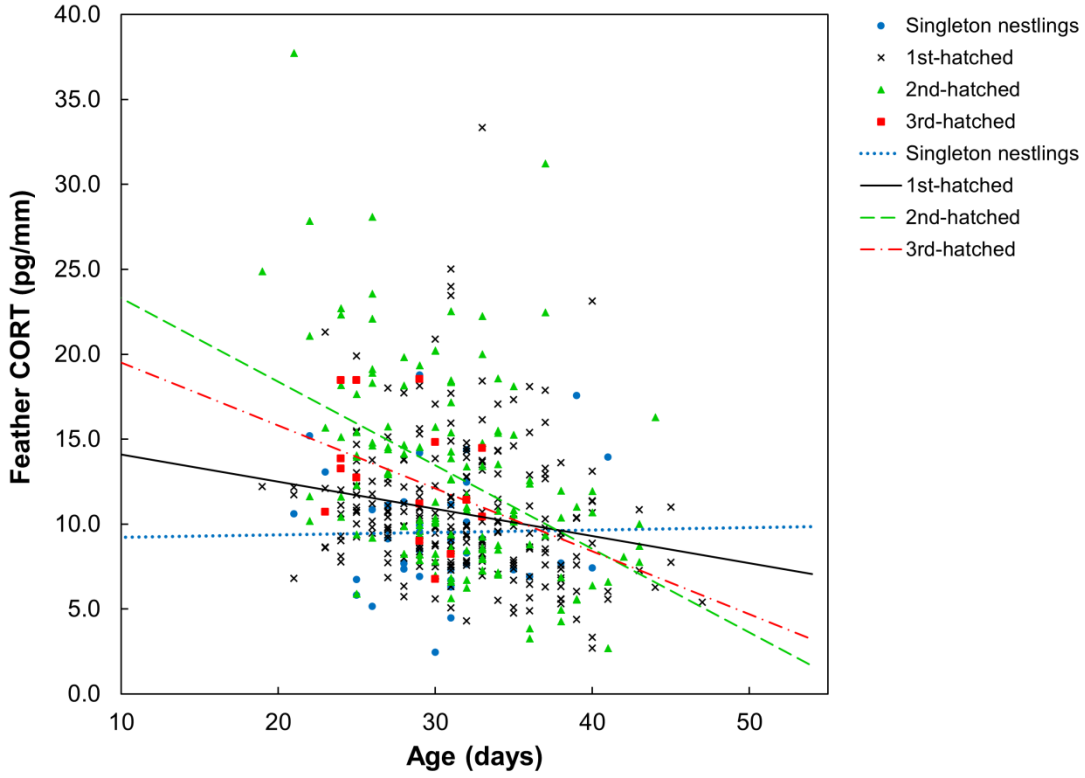

Fig. 3. Effect of the interaction between age and brood order on feather corticosterone (CORT) levels of nestling black kites. Chicks from single broods exhibited constant feather CORT levels throughout development, whereas those of nestlings hatched in multiple-chick broods declined gradually with increasing age.

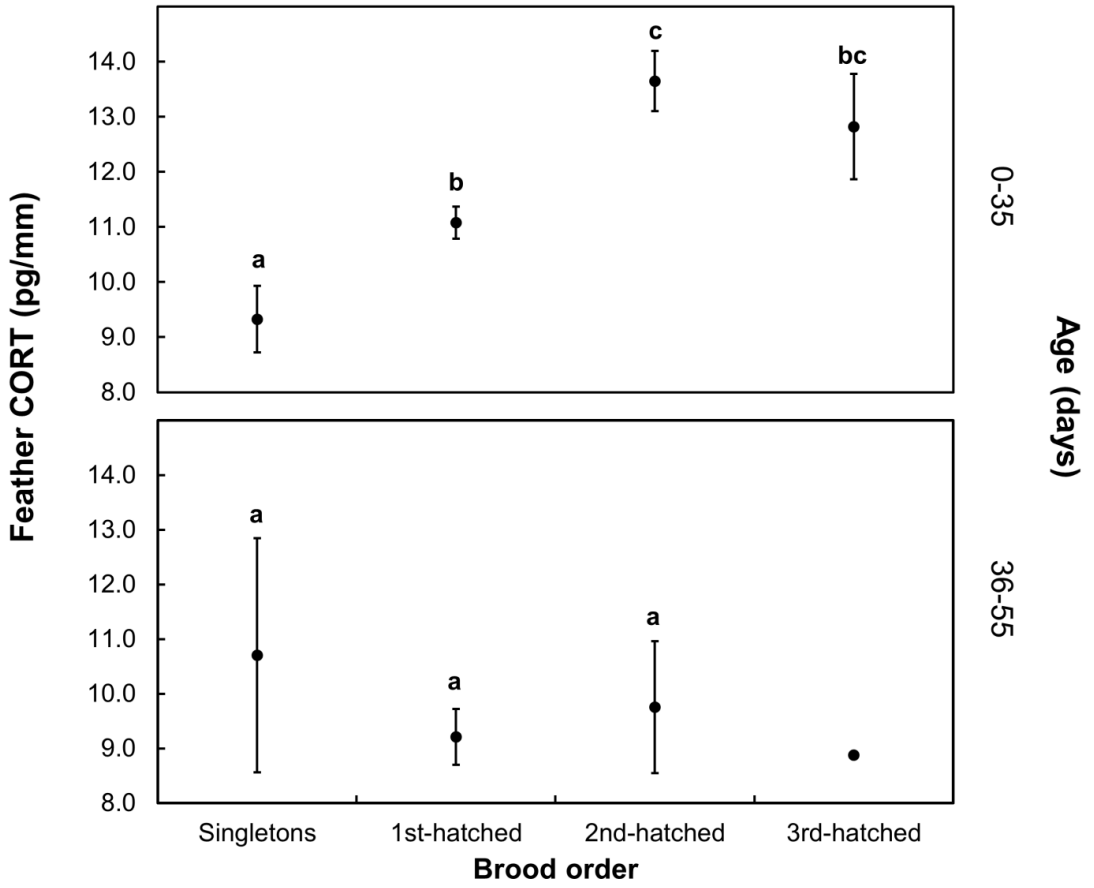

Fig. 4. Differences in feather corticosterone levels between singletons, senior and junior nestlings during early development (first 35 days of age, upper panel), and late development (from 36 days of age until fledging, lower panel). Different letters signify significant differences among groups (Tukey's HSD post-hoc test $P < 0.05$).

DISCUSSION

Our results indicate that CORT-F levels in developing black kites are associated with mean ambient temperatures experienced during the period of feather growth, with body condition at the time of sampling and with nestling dominance status during the early developmental stages.

In endotherms, the ability to maintain homeostasis in core body temperature at minimum cost is limited to a narrow range of ambient temperatures known as the zone of minimum metabolism (ZMM; Etches, Jonh & Verrinder Gibbins 2008). As temperatures decrease below the ZMM, allostasis becomes increasingly costly, for individuals must invest energy in the activation of physiological mechanisms aimed at increasing heat production (Scholander et al. 1950; Etches et al. 2008). Our study model is geographically distributed in warm environments of tropical and temperate regions, clearly avoiding cold climates (Del Hoyo, Elliott & Sargatal 1994). The negative association between ambient temperatures and CORT-F may thus indicate that nestlings exposed to colder temperatures during development required a higher investment of energy for thermogenesis. The latter process requires increasing basal metabolic rates and the use of body stores for fuel, and these two processes are likely mediated by elevated circulating CORT levels, as has been observed in other species (Palokangas & Hissa 1971; Mujahid 2010; Wack et al. 2012). Our results are, therefore, consistent with the idea that nestlings raised during the coldest periods of the breeding season experienced a higher allostatic load for thermogenesis that translated into higher CORT-F levels (likely reflecting elevated plasma CORT levels; Romero, Reed & Wingfield 2000; Frigerio et al. 2004; Lobato et al. 2008). At the same time, the decrease in CORT-F levels with

increasing temperatures could also be reflecting an effect of weather on food supply. Growth rates in nestling Black kites have been positively related to increasing ambient temperatures (Hiraldo et al. 1990). To explain this relationship, the authors suggested that high temperatures could be facilitating adult foraging by promoting the creation of thermals. Under such scenario, the decrease in CORT-F levels would be a consequence of increased parental provisioning rates, supporting a large body of evidence that optimal feeding conditions decrease circulating CORT levels in taxonomically different species (Núñez de la Mora, Drummond & Wingfield 1996; Schoech, Mumme & Wingfield 1997; Moore et al. 2000; Kitaysky et al. 2001a; Romero & Wikelski 2001).

In line with this argument, our results also showed that CORT-F was negatively related to nestling body condition, suggesting that levels of CORT measured in feathers increased as nutritional status worsened. However, interpreting the biological significance of CORT-F levels can be tricky and requires some prior knowledge of the way in which different species respond to food limitations at the time of allocating resources preferentially to the different growing tissues. In a first approach to understanding such mechanisms, Bortolotti et al. (2009) investigated the way in which CORT levels varied along the length of individual feathers in six avian species in which feather formation rates were known to be approximately linear throughout growth. The patterns observed led the authors to the idea that CORT is most likely incorporated into feathers in a time-dependent, rather than mass-dependent manner, such that equal length portions of feather, grown assuming identical plasma CORT concentrations, would be expected to show similar hormone levels regardless of mass (i.e. wider and thus heavier segments become more diluted). More recently, however, variations in feather density, as opposed to differences in mass attributable to width, have been

shown to exert an important effect on CORT deposition. The latter appears to be particularly relevant in species which respond to nutritional stress by drawing resources away from feather growth, because such mechanism results in thinner and therefore lighter feathers containing less blood-derived materials (including CORT) than would be predicted from their respective concentrations in plasma. This was recently demonstrated by Patterson et al. (2015), who showed that feathers of Caspian Tern chicks raised under a nutritionally restricted diet were shorter, less dense and contained lower CORT levels than feathers of chicks fed ad lib. Thus, although nutritional stress and decreased body condition typically increase plasma CORT (both baseline and stress-induced; Kitaysky et al. 2001a; Pérez-Rodríguez et al. 2006; Poisbleau et al. 2010, but see Müller, Jenni-Eiermann & Jenni 2010), concurrent CORT-F elevations (in pg/mm) should only be expected if both feather growth rates and density remain unaffected by nutritional status (Will et al. 2014). Black kites make good study models in this sense, because nestlings respond to dietary restriction by investing resources preferentially in feather growth to maintain constant elongation rates in detriment of body mass gain and bone formation (Hiraldo et al. 1990; Viñuela & Bustamante 1992). This, combined with the fact that nestlings in poor body condition exhibited higher CORT-F levels than those in good condition (contrary to what would be expected if feather density was being negatively affected by poor nutritional status), suggests that CORT-F levels may be reasonably assumed to reflect levels of circulating CORT during feather growth. In principle, temporary (short-term) CORT rises could benefit nestlings in several distinct ways: (1) by promoting gluconeogenesis, the mobilization of lipid stores and the inhibition of fat storage (all of which increase the availability of blood glucose to support the maintenance of essential body functions; Sapolsky et al. 2000); (2) by promoting the catabolism of muscle

protein, which can serve as an alternative source of energy once fat stores have been depleted. This strategy would be particularly valuable to Black kite nestlings, since the species is heavily reliant on temporary and unpredictable pulses of overabundant resources (Viñuela & Veiga 1992; Sergio et al. 2011a); and (3) by facilitating behavioural responses aimed at increasing food intake. Whereas in adults this can be achieved by intensifying foraging behaviour (Angelier et al. 2007), nest-bound, non-precocial chicks display functionally equivalent responses also related to CORT levels, such as begging (Kitaysky, Wingfield & Piatt 2001b; Kitaysky et al. 2003) and sibling aggression (Kitaysky et al. 2003).

The Developmental Hypothesis posits that in order to avoid the deleterious effects of chronic exposure to elevated CORT levels, HPA axis maturation should parallel the development of the physiological and behavioural abilities allowing organisms to cope with or overcome potential perturbations (Schwabl 1999; Sims & Holberton 2000; Blas et al. 2005). However, contrary to predictions, we found that singleton nestlings displayed low and relatively constant CORT-F levels throughout development, while in nestlings from multiple-chick broods CORT-F actually declined with age. Moreover, for the latter, the effect of age interacted with rank position within the brood hierarchy. Thus, while senior chicks exhibited lower CORT-F than their siblings at young ages, these differences gradually diminished as birds grew older until they eventually disappeared in the late nestling stages. Altogether, the described patterns suggest an effect of the social environment on CORT-F levels, likely reflecting the increased allostatic load imposed by the oldest, dominant chick within each brood on its younger siblings during the establishment of brood hierarchies, and support the role of CORT in the regulation of submissive behaviour (reviewed by Leshner 1980). For instance, in mice, individuals injected with high doses of CORT (Leshner et al. 1980), or those allowed to respond

naturally to adrenocorticotropic hormone (ACTH) injection through CORT secretion (sham-adrenalectomised mice; Leshner & Politch 1979), surrendered after a significantly lower number of attacks than either control or adrenalectomised mice respectively. In a different experiment, plasma CORT increased following fights in both dominant and subordinate competitors, yet the magnitude of the increase was shown to be larger and longer-lasting among losers, implying a higher energetic cost of social stress on the latter compared to winners (Leshner 1980). Among adult Black kites, stress-induced CORT levels are higher in floating than in breeding males, while females show the opposite pattern (Blas et al. 2011). The latter findings support the idea that deferred reproduction is socially imposed by dominant conspecifics, and CORT responses reflect the sex-specific roles during competition for territories in socially monogamous species. More broadly, a review on mammal and avian social systems (Creel 2001) exposed a trend for subordinate individuals to exhibit higher baseline CORT levels in: (1) situations where subordinates cannot avoid or escape aggressions from dominants (e.g. nest-bound chicks); or (2) stable hierarchies, where dominants are rarely challenged and therefore do not need to invest in frequent fights to assert their status. In line with this idea, Black kite brood hierarchies develop during the first weeks of life, with fights typically starting on day 1 post-hatch and peaking during the third week (Viñuela 1999). However, once the hierarchy is established, it is practically always maintained for the rest of the rearing period with minimum agonistic interaction (Viñuela 1999). Such stability in dominant-subordinate relationships is a common trait in avian broods, where losers gradually learn to "accept" their role in the hierarchy by submitting increasingly faster to the attacks from dominant siblings (thus avoiding the costs of continuous escalated fights; Leshner 1980; Drummond & Osorno 1992). Moreover, social challenge dynamics would also explain why

singleton chicks, never experiencing sibling aggression, exhibited consistently low CORT-F levels throughout their nestling period, thus acting as sort of "control" individuals not subjected to social pressures.

Alternatively, age-related declines in CORT-F could be reflecting the gradual disappearance (through mortality) of nestlings subjected to strong allostatic loads during development. Such scenario, however, is unlikely given that negative selection would be expected to drive a similar age trend in CORT-F levels of singleton chicks, especially since one-chick broods are mainly associated with young first-time breeders which typically experience difficulty to provide adequately for their chicks (Blas, Sergio & Hiraldo 2009). As our results here suggest, poorly fed chicks are more prone to suffer greater allostatic loads as a result of their lower energy stores, so negative selection would be expected to exert a particularly strong impact on them. We suggest, nevertheless, that in order to confirm or reject this hypothesis, future analyses should contemplate cutting up feathers of nestlings that reach fledging age into proximate and distal halves (representing the late and early nestling periods respectively) and analysing CORT-F levels separately in each one to determine whether they are significantly different (offering support to the social challenge hypothesis) or similar (supporting the negative selection hypothesis).

In summary, our results support the growing appreciation that the CORT content of feathers varies in parallel with environmental, physiological and social challenges, and may thus be used to infer allostatic loads. Detectable CORT levels have been reported in the feathers of all avian species analyzed so far (ca. 35 species), representing a diverse range of orders, habitats, ecological contexts, body sizes, trophic positions and behaviours (e.g. Bortolotti et al. 2008; Koowenberg et al. 2013; Legagneux et al. 2013; Meitern et al. 2013). Such generalized trait

underscores the potential for conducting similar analyses to the ones applied in this study on other wild birds, thereby opening the door further to the use of feathers as widespread indicator tools in conservation. For instance, comparing CORT-F levels across individuals or populations could allow to identify relevant environmental pressures and facilitate the establishment of conservation priorities. CORT-F analyses could also serve to detect the factors that are most detrimental for threatened species, promoting a more efficient design of recovery programs. Finally, extending the feather sampling protocol over the course of several years could prove a simple but effective method for tracking and evaluating the success of implemented conservation measures, as well as to monitor the emergence and impact of new, potentially detrimental perturbations over time (e.g. construction of nearby roads or wind farms, habitat destruction, climate change).

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DATA ACCESSIBILITY

Access to the relevant data for this article will be restricted during a two-year embargo period. Data will be publicly available in the Dryad Digital repository at http://dx.doi.org/10.5061/dryad.ch06v after the embargo expires.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article.

Table S1. Summary data for the response and predictor variables used in the statistical analysis of this study.

CAPÍTULO 3

Age-related patterns in feather corticosterone levels reflect lifetime variation in environmental challenges

Age-related patterns in feather corticosterone levels reflect lifetime variation in environmental challenges

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ABSTRACT

In vertebrates, exposure to challenges to the homeostasis of physiological systems, such as energy demanding or stressful conditions, triggers a cascade of endocrine secretions involving the hypothalamic-pituitary-adrenal axis. In birds, this results in the release of the hormone corticosterone (CORT) into the bloodstream, which induces behavioural and physiological changes that mediate allostasis. Both the type and number of challenges an individual is likely to encounter in life, as well as how they are dealt with, are expected to vary in an age-related manner encompassing the progressive acquisition and improvement of skills and the escalation of social hierarchies. Here we investigated whether the levels of CORT deposited in feathers during regular moult reflected the expected lifelong progression of energetic challenges in a long-lived raptor, the black kite (*Milvus migrans*). Feather CORT values were highest in the youngest birds, gradually declined to reach minimum levels in birds 7-11 years old and then increased again among the oldest, senescent birds (≥12 years old). This pattern closely mirrored the age-related changes in reproductive success and survival rates previously reported for this population, suggesting that feather CORT levels reflected the challenges and most vulnerable periods experienced by these birds as they proceeded through life. In addition, feather CORT levels were negatively related to body size, implying that larger birds either experienced fewer homeostatic challenges, or were better able to cope with them. Altogether, our results highlight the potential of using feather CORT measures as a complementary, non-invasive tool in conservation to assess the relative health of populations.

INTRODUCTION

Throughout their lives, animals continuously face numerous threats and challenges which can ultimately compromise their performance and survival, including sibling competition (Viñuela 1999; Hofer and East 2008), harsh weather (Brown and Brown 2000), food shortages (Dawson and Bortolotti 2000; Romero and Wikelsi 2001), predation (Tapper et al. 1996; Lettink et al. 2010), conspecific interactions (Essington et al. 2000; Stokes and Boersma 2000) or costly programmed events in the life-history of species (e.g. migration, reproduction; Menu et al. 2005; Newton 2006; Cox et al. 2010). In birds, exposure to any of these energy demanding situations can trigger the activation of a physiological cascade of events involving the hypothalamic-pituitary-adrenal (HPA) axis, and resulting in the release of the steroid hormone corticosterone (CORT) into the bloodstream (Wingfield et al. 1982; Romero and Remage-Healey 2000). CORT rises play a fundamental role in the mediation of allostasis (the process of maintaining homeostasis through change) by promoting a suite of behavioural and physiological responses aimed at coping with challenges or escaping perturbations in the best condition possible (McEwen and Wingfield 2003; Blas 2015). These include, for instance: (1) diverting energy resources away from non-immediately-essential functions such as reproduction or territoriality (Wingfield and Silverin 1986; Angelier et al. 2009); (2) increasing gluconeogenesis and the mobilisation of fat deposits to support heightened physical activity (e.g. during sustained or prolonged antagonistic encounters) by delivering a rapid energy boost to cells (Challet et al. 1995; Breuner et al. 1998); (3) increasing foraging or nestling begging rates, to help animals maintain body condition in response to food shortages or

during energetically demanding periods (e.g. migration or brood rearing; Astheimer et al. 1992; Kitaysky et al. 2001; Angelier et al. 2007a); or (4) progressive adjustments in the physiological state of organisms, directed to meet predictable fluctuations in the external and internal demands (collectively known as "allostatic load"; McEwen and Wingfield 2003) associated with routinely day-to-day activities or seasonal changes in the environmental conditions (e.g. higher metabolic rates to increase internal heat production in response to decreasing night or winter temperatures; Palokangas and Hissa 1971; Landys et al. 2006; Wack et al. 2012; see Blas 2015 for a review).

As individuals grow older, both the type and number of challenges they encounter, as well as the way in which these are coped with, are likely subjected to progressive changes. Younger individuals, for instance, typically accumulate experience and improve skills through repeated practise over the years, thus potentially reducing the impact of unpredictable perturbations or "stressors", as well as the energetic requirements to perform ordinary daily activities. Ageing is also frequently associated with changes in habitat or space use (Ferrer 1993; Caro et al. 2011), or adjustments in behavioural and phenotypic traits (e.g. migration strategies or body size; Mueller and Berger 1976; Mueller and Berger 1979; Sergio et al. 2014) that often lead to differential exposure to threats and changes in social status (Richner 1989; Sergio et al. 2011b). The expected resultant effects of such age-specific changes on CORT levels might be especially evident in long-lived species (where learning and experience are likely more relevant and prolonged), and in saturated populations (where competition for resources is substantial and conflict resolution is typically mediated by age or phenotypic dominance).

Despite the growing number of studies assessing adrenocortical function in wild vertebrates, only a handful so far have examined age-related variation in CORT levels in long-lived animals. Furthermore, most of these studies have: (1) concentrated on marine avian species that breed colonially; (2) focused almost exclusively on the breeding portion of the population (thus neglecting non-breeders); (3) assessed plasma CORT levels (which offer a rather instantaneous snapshot view of an individual's physiological state at the moment of sampling), and (4) reported conflicting results in different species, highlighting the fact that changes in CORT levels and associated stress responses are probably complex and context specific (Angelier et al. 2006; Heidinger et al. 2006). Consequently, to achieve broader taxonomic representation and generality, there is an urgent need of complementary information on: (*i*) the HPA-axis physiology of long-lived species differing in general ecology from marine birds; (*ii*) changes in CORT profiles spanning the entire lifespan of a species (i.e. comprising all ages and stages of the life cycle, including both breeders and non-breeders), and (*iii*) integrated measures of adrenocortical activity encompassing longer-term variation in exposure to both predictable and unpredictable challenges, which may offer a complementary and thus more comprehensive picture of the changes that animals experience in their allostatic loads as they proceed through life.

Here, we explore the effect of age and traits associated with social status (e.g. body size and body condition) on the feather CORT profiles (CORT-F) of a medium-sized, long-lived raptor: the black kite (*Milvus migrans*). The study population has been intensely monitored for the past 30 years, providing detailed long-term, life-history information for a large number of marked individuals of known age. In addition, the population is currently saturated at more than 500 breeding pairs, with densities of up to 10 pairs km^{-2} in some areas (Sergio et al.

2011a). Such crowding has favoured the establishment of a system of social subordination for access to food and nesting sites (Sergio et al. 2009a) that is reflected in sex- and status-specific patterns of adrenocortical response to stress (Blas et al. 2011). In this study, we test the hypothesis that CORT profiles vary over the life span of kites in a manner that reflects the most energetically challenging episodes in their lives. Previous studies conducted on this population reported parallel, age-structured variation in breeding performance and survival rates (Blas et al. 2009; Sergio et al. 2011b), which served to highlight the most vulnerable stages of the species' life cycle. Based on such previous knowledge, we examined whether variation patterns in CORT-F levels would be best characterised by: (a) chronological age (in years); or (b) specific age-intervals that coincide with major demographic stages in the life cycle of the species (Blas et al. 2009; Sergio et al. 2011b; Sergio et al. 2014). These are: (Age 1 year old) individuals in their first year of life, when the major mortality bottleneck occurs in the population (less than half the animals survive to the next year; Sergio et al. 2011b); (Age 2) juvenile non-breeders, still incapable to hold a territory because of their inexperience and sub-dominant social status; (Age 3-6) young adults that have accumulated enough experience and social status to start recruiting into the breeding population; (Age 7-11) adults in their prime age of maximum reproductive output and survival; (Age ≥ 12) oldest, senescent individuals, characterised by lower breeding performance and survival.

MATERIALS AND METHODS

Study species

The black kite is a medium-sized, migratory raptor present in most temperate and tropical regions across the Paleartic, Indomalasia and Australasia. The maximum recorded longevity in our population is 28 years. Such a long lifespan affords individuals the possibility to gauge the potential costs and benefits of reproduction and delay their recruitment into the breeding population if conditions are not favourable (Sergio et al. 2009a). As a result, age of first breeding is variable, and can lie anywhere between 1-7 years (mean = 3.5, Blas et al. 2009). Our study population in Doñana National Park consists of ca. 500 breeding pairs plus an important pool of juveniles and non-territorial adults (collectively known as "floaters") estimated at 400-500 individuals. Both segments of the population coexist in the same space and share the same habitat preferences, as shown by telemetry data (Tanferna et al. 2013).

Sample collection

We trapped 143 known-age kites (1-24 years-old) across six years (2007-2012), using cannon nets baited with carrion at the beginning of the reproductive season. For each individual we measured body mass (to the nearest 0.5g), tarsus length (to the nearest 0.1mm), and wing and tail length (both to the nearest 0.1cm). Three upper tail covert feathers were collected from each bird, placed in plastic zip bags and stored at -21°C for subsequent determination of CORT levels. Tail coverts were chosen because their collection was judged not to harm or impair the birds in any way. These body feathers have an estimated natural growth period of around

25 days (Ontiveros 1995) and are moulted on a yearly basis in the African winter quarters, with the first moult taking place during the first winter after fledging (Baker 1993). All trapped birds were sexed by molecular analysis of a blood sample (Ellegren 1996).

Corticosterone analysis

The analysis of CORT-F levels is a relatively novel technique (Bortolotti et al. 2008) which relies on the deposition of circulating glucocorticoids in the keratin structure of feathers as they grow, for it is during this period only that feather cells become vascularized. CORT-F levels thus provide an integrated measure of HPA-axis function, likely reflecting both the total baseline and stress-induced CORT levels experienced by an individual over the relatively ample period of 3-4 weeks encompassing feather growth (Fairhurst et al. 2013; Jenni-Eiermann et al. 2015). In addition, feathers possess several useful features which make them particularly appealing for field endocrinology studies (Blas 2015). Firstly, the individual historical record of CORT exposure is available to the researcher for collection for as long as the feather remains on the bird (typically 1 year, until the next molt). Secondly, unlike hair, feathers do not appear to incorporate CORT from exogenous sources like, for instance, preen oils (Thieme et al. 2003; Bortolotti et al. 2008). Lastly, CORT-F has been shown to be highly stable over time and resistant to degradation upon exposure to high temperatures (Bortolotti et al. 2009). This is a particularly important feature, given that feathers are often not collected until months after they have been grown (especially in migratory species like black kites, which initiate and complete their moult at their wintering grounds).

Extraction of CORT from feathers was carried out using a methanol-based technique (see Bortolotti et al. 2008: *Supplementary Appendix S1*). First, the length of each feather was measured, excluding the calamus which was removed. Then, each feather was cut into pieces of < 5 mm^2 and placed in a glass vial to which 10 ml of methanol was added (HPLC grade, VWR International, Mississauga, ON). The vials were placed in a sonicating water bath at room temperature for 30 min, followed by incubation at 50 °C overnight in a shaking water bath. Using vacuum filtration, the methanol containing the hormones was separated from the feathers, washing vials with additional methanol and adding it to the original methanol extract. Vials with the methanol extract were placed open in a 50 °C water bath in a fume hood under air for evaporating until they were completely dry. When the evaporation of the samples was completed, the extract residues were reconstituted with 1200 µl of phosphate buffer system (PBS; 0.05M, pH 7.6) and frozen at -20 °C until CORT was measured by radioimmunoassay (RIA). Seven extraction processes were carried out in total. To determine the recovery of the extraction, three feather samples per batch were spiked with a small amount (~5000 CPM) of [^{3}H] corticosterone and the percentage of radioactivity recovered was assessed. Greater than 90% of the radioactivity was recoverable in the reconstituted samples. CORT concentration was determined using RIA, as described in Wayland et al. (2002) and Bortolotti et al. (2008). Measurements were performed on duplicate reconstituted extracts. Serial dilutions of feather extracts were parallel to the standard curve. Antiserum was purchased from Sigma Chemicals (product C8784, lot number 090M4752) and purified CORT for standards were purchased from Amersham Bioscience. Assay variability was determined as the % coefficient of variation (CV) resulting from repeated measurement of samples spiked with a known amount of CORT in each assay. We performed 12 assays with a mean intra-

assay CV of 4.9 %, and inter-assay CV of 17.4 %. We expressed CORT-F values as pg CORT mm^{-1} feather because deposition of CORT into growing feathers is hypothesized to be time dependent, not mass dependent, and feather length is the most valid estimate of unit time of feather growth (see Bortolotti et al. 2008; Bortolotti et al. 2009; Bortolotti, 2010; Jenni-Eiermann et al. 2015). Hormone analyses were performed in the Department of Biology, University of Saskatchewan (Canada).

Statistical analyses

Body size was estimated by means of a Principal Component Analysis (PCA) on wing, tail and tarsus length. The analysis extracted a single major component (PC1) accounting for 57% of the variation, with high positive loadings for both wing and tail length. Body condition was estimated as the residuals from the ordinary least squares regression of body mass on PC1 scores following Schulte-Hostdde et al. (2005).

We used Generalized Linear Mixed Models (GLMM; Zuur et al. 2009) to test the effect of explanatory variables on observed CORT-F levels. All tests were implemented in R 2.15.2 (R Development Core Team 2009). The saturated model included: (1) CORT-F (pg mm^{-1}; ln-transformed) as the dependent variable; (2) sex and age class as potential categorical predictor variables; (3) body size, body condition and age in years as continuous predictor variables; and (4) both sampling year and individual identity as random effects, to account for year-to-year variation in environmental conditions and pseudoreplication, resulting from recapturing some individuals in different years (n = 4). Given the obvious association between age and age category, models including either variable were built separately.

Whenever age in years was the variable under consideration in the model, the effect of its squared term was also tested in order to assess non-linear relationships between CORT levels and age that might resemble age-associated patterns in survival and reproductive success previously reported for this population. In addition, based on the available literature on the biology of the species (Sergio et al. 2007a; Blas et al. 2011), the following interactions were also explored: sex x age/age category; sex x body size and sex x body condition.

In order to detect and correct for potential collinearity problems between independent terms in the GLMM analyses, we previously tested for correlations between predictor variables as part of the data inspection process. Since body size was positively associated with age, we used the residuals of body size on age or age category in the corresponding GLMM analyses. Model simplification was implemented through a backward stepwise procedure of model selection whereby the fit of the saturated model, in terms of residual deviance, was compared to that of a series of reduced models in which each one of the terms was excluded sequentially. After all possible comparisons were made, the least significant term (i.e. the one producing the smallest non-significant increase in deviance), was removed from the model. The process was then repeated until only significant terms remained in the minimum adequate model (Zuur et al. 2009). Best model selection between competing final models was based on final Akaike's Information Criterion (AIC) scores. Generally, a ΔAIC >2 between two candidate models is considered to offer substantially more support to the model with the lowest AIC (Burnham and Anderson 1998).

RESULTS

The best final model retained the independent effects of age category (χ^2 = 18.07, P = 0.001) and body size (χ^2 = 6.19, P = 0.013): CORT-F levels were highest in one year old birds, declined progressively through the successive age stages until reaching minimum levels at age 7-11, and increased again in senescent adults (≥12 years old, Fig.1a). The negative slope associated with body size (estimate = -0.06 ± 0.02 SE) meant that, within each given age category, smaller individuals showed significantly higher CORT-F levels than larger birds (Fig. 1b).

The second best final model retained the same two variables, age and body size, although this time the former entered as a continuous covariate and in its quadratic form (body size: χ^2 = 11.56, P < 0.001; age^2: χ^2 = 5.81, P = 0.016). Both the significance of the squared term, indicative of a non-linear relationship between CORT-F and age, as well as the magnitude and sign of the slope of the association between CORT-F and body size (estimate = -0.08 ± 0.02 SE) were in accordance with the best model described above However, the latter (that including age category and body size) provided the most accurate description for variability in CORT-F levels, both in terms of the lowest overall AIC score (7.085 vs 11.679 for first and second best models respectively) and ΔAIC.

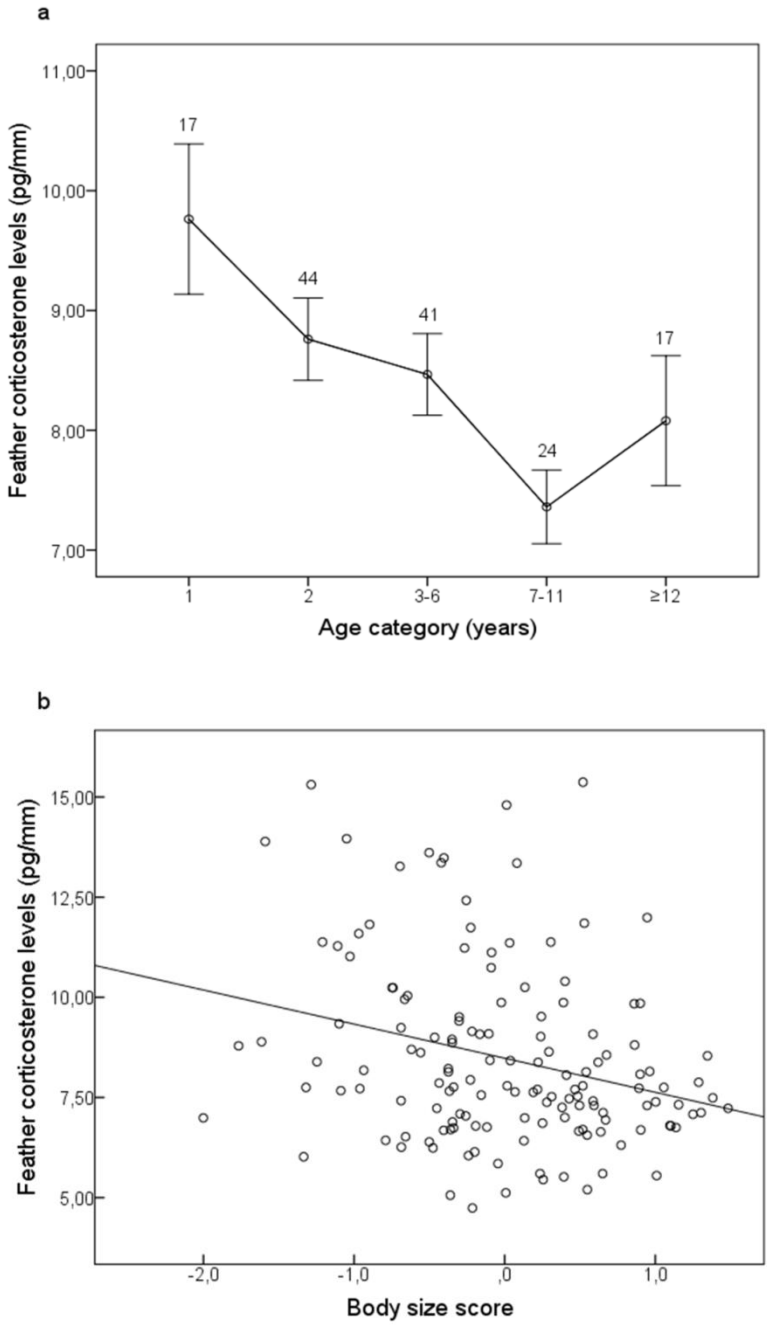

Fig. 1 Changes in feather corticosterone levels of black kites (n = 143) in Doñana National Park: (a) across the whole life span of the species, and (b) in relation to body size (corrected for age category). Values in panel (a) are expressed as means ± 1 SE. Numbers above error bars represent sample size for each age category

DISCUSSION

Our analyses, exploring age-related variation in CORT-F levels throughout the whole life span of a wild, long-lived raptor, suggest that the average allostatic load experienced by these birds is strongly dependent on their current life-stage and overall body size. CORT-F levels were highest among one year old birds, gradually declined across age classes to reach minimum titres in 7-11 year old (prime age) individuals, and finally increased in the oldest segment of the population (ages ≥12, Fig. 1a). These results complement the very scarce available information on life-long, ontogenetic patterns of CORT secretion in wild vertebrates, to our knowledge only available for six other wild animal species (Jessop and Hamman 2005; Angelier et al. 2006; Heidinger et al. 2006; Angelier et al. 2007b; Angelier et al. 2007c; Wilcoxen et al. 2011). The reported pattern, which mirrored age-dependent changes in survival and reproductive success previously described for this population (Blas et al. 2009; Sergio et al. 2011b) can be interpreted in terms of the sequence of energetic challenges that individuals face as they progress through life. In this context, the elevated CORT levels found in the feathers of one year old birds likely reflected the concerted action of higher exposure to novel stimuli and energetic challenges associated with their overall lack of experience. Novelty exposure *per se* typically induces plasma CORT rises in animals (Richard et al. 2008; Beerling et al. 2011), while repeated exposure to the same type of stressor through acclimation has been shown to gradually reduce the magnitude of HPA activation (Grissom and Bhatnagar 2009). Thus, together, these two mechanisms could partly explain why CORT-F levels were highest among these youngest kites compared to the rest of age groups. On top of this, one year old birds typically show

poorer foraging skills compared to older individuals (Marchetti and Price 1989; Watson and Hatch 1999; MacLean 1986; Jansen 1990; Desrochers 1992). Since reduced food quality and quantity triggers rises in plasma CORT levels (Hector and Harvey 1986; Kitaysky et al. 1999; Kitaysky et al. 2007), poorer foraging efficiency could also ultimately translate into higher CORT-F titres (e.g. Will et al. 2015). The latter effect could be further boosted by social subordination imposed by older individuals through aggressive behaviour, often displacing young birds from easily accessible food resources such as carrion (Sergio et al. 2011c). Finally, the first few months of life represent a period of heightened energetic demands for black kites as they encompass growth and development, fledging, parental independence and first fall migration. Despite the fact that all these events occur months to weeks before winter feather moult, the expected plasma CORT elevations required to meet such increased allostatic load could potentially translate into higher CORT-F levels through carry-over effects (Marra and Holberton 1998; Sedinger et al. 2011), providing a further plausible explanation for the recorded peak rates of CORT deposition in the feathers of one year old birds.

In turn, the strong selective pressures experienced by kites during their first year of life implies that only a small percentage of individuals will survive to the following year (Sergio et al. 2011b). Birds reaching two years of age, therefore, represent a pool of higher-quality individuals that are capable of accumulating enough experience during their first year to improve their chances of survival and overcome the mortality bottleneck. On account of this, we suggest that the progressively lower CORT-F values found in each of the successive age groups could respond to the continuous enhancement of key skills (e.g. foraging, fighting, predator evasion), which would allegedly facilitate coping with the challenges and energy demands of every-day activities.

In agreement with this line of argument, previous studies examining lifelong changes in migratory performance in this population through satellite-telemetry data have demonstrated the occurrence of age-related adjustments in a number of migration components associated with higher fitness (Sergio et al. 2014). For instance, tagged kites exhibited a progressive, marked tendency to advance the departure date of their spring migration with increasing age. As a result, older birds obtained a significant time advantage over juveniles (1-2 year olds) and young adults (3-6 year old birds) that allowed them to make use of energy minimization strategies such as lower flight speeds, longer stop-overs and fewer travelling hours per day. At the same time, younger kites were found to be more strongly affected by crosswinds *en route* than older individuals, suggesting that the ability to cope with environmental conditions is a skill that requires time and experience before being fully mastered by these birds. More importantly, the fact that individual birds were subjected to long-term (~3 years) monitoring enabled the authors to ascertain the contribution of longitudinal improvements in performance in shaping the above lifetime patterns, thus supporting the idea that the ontogeny of CORT-F profiles reflects how the way in which individuals perceive and deal with environmental, social and physiological challenges changes as they grow older.

Nevertheless, there are other non-exclusive explanations for the observed age-related decline in CORT-F levels which require consideration. First of all, the nature of the dataset used in these analyses was cross-sectional. The implications of using this type of data mainly relate to the inability to tease apart the effects of longitudinal (within-individual) changes in HPA function from the action of population-level selective mortality in producing the pattern of Figure 1a. Thus, decreasing values of CORT-F with advancing age could also be attributable to the action of natural selection favouring phenotypes that show reduced HPA axis

responsiveness to environmental stressors, as suggested in other long-lived birds (Blas et al. 2007). At first glance, the latter hypothesis might seem contradicting, given that a robust physiological response to a stressor is generally assumed to be necessary to successfully deal with the source of stress. Though this would logically bestow some adaptive value to acute CORT levels, direct evidence in support of this idea is not overwhelming in the literature (Hau et al. 2010; reviewed by Breuner et al. 2008). In contrast, there seems to be a growing number of studies that report detrimental consequences and fitness costs associated with long-term, chronically elevated CORT levels. In particular, sustained high concentrations of circulating glucocorticoids have been found to lead to immunosuppression (Sapolsky et al. 2000), impaired cognitive abilities (Kitaysky et al. 2003), reduced developmental growth and body condition (Cabezas et al. 2007; Müller et al. 2009), lower offspring production (Ellenberg et al. 2007) and poorer survival rates (Romero and Wilkelski 2001; Koren et al. 2012). Thus, it could be expected that reactive individuals, i.e. those that under identical environmental perturbations experience higher adrenocortical responses, could be suffering a selective disadvantage against less responsive individuals, which should favour their early disappearance from the population (see e.g. Blas et al. 2007). Alternatively, age-related CORT-F declines could be mechanistically driven by an ontogenetic decrease in the capacity of the adrenals to secrete CORT or to respond to adrenocorticotropin hormone (ACTH; Heidinger et al. 2008).

Although we cannot discard the contribution of the latter two mechanisms, we nevertheless find them unlikely to be the main factors driving the lifelong changes in CORT-F values observed in this study, as they would be unable to explain the increase in CORT-F levels observed in the oldest age group. Instead, such final upward trend was again remarkably consistent with the previously

described declines in reproductive output and survival rates attributed to the onset of senescence (Møller and Lope 1999; Catry et al. 2006; Sergio et al. 2011b). Senescence, otherwise known as ageing, is defined as the progressive degradation of the phenotype with increasing age, leading to the deterioration of essential functions such as decreased foraging skills, fertility or poorer immune function (Newton and Rothery 1997; Kirkwood and Austad 2000). The higher CORT-F levels found in birds belonging to the oldest age category could therefore be reflecting the way in which this loss in competitiveness renders old individuals increasingly vulnerable again to environmental, social and physiological challenges.

Interestingly, our results agree with previous studies reporting similar declines in baseline plasma CORT levels between first-time breeding and more experienced black-browed albatrosses (*Thallasarche melanophris*), followed by a significant rise in CORT titres among the oldest birds accumulating 13 or more reproductive attempts (Angelier et al. 2007b). More importantly, the latter study also found that chronological age *per se* was not a good predictor of CORT values, but that, similarly to our findings, variation in CORT levels was instead best characterised by three important life-history stages, namely, recruitment/first breeding, adulthood and senescence. Such results suggest the possibility that the lifelong curve in CORT-F levels outlined in Figure 1a could be a generalized pattern linking HPA axis function to the life history of long-lived species.

Finally, we found a weaker but nevertheless significant negative relationship between CORT-F levels and body size (Fig. 1b), implying that, in general, larger birds were likely less exposed to challenges or were better able to cope with them compared to smaller individuals of the same age category. Other studies, including previous analyses on this kite population, have linked larger body size to earlier

arrival dates from migration (Lindström et al. 1990; Sergio et al. 2007b), higher competitive abilities in securing high-quality mates and territories (Weatherhead and Boag 1995; McDonald et al. 2005), better reproductive rates (McDonald et al. 2005; Sergio et al. 2009b), and a higher chance of winning intraspecific contests over food or against territorial intruders (Sergio et al. 2007b). This generalised advantage of large size on such diverse fitness-related traits is bound to contribute towards creating a less hostile and energetically demanding environment, which would consequently reduce the CORT-F titres of larger birds.

In summary, the present study provides a complete screening of the variation in CORT-F levels along the entire life-time of a wild, long-lived vertebrate, suggesting that this measure accurately reflects the most sensitive and energetically challenging periods for birds as they proceed through life. These results thus highlight the potential of using CORT-F measures to generate rapid, reliable and easily interpretable results for a large number of individuals and in a non-invasive manner, which may serve, for instance, to compare the physiological state of populations, of different sectors of the same population or to monitor changes in allostatic load over time. Such features are particularly crucial when dealing with species in peril that demand immediate conservation action.

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CAPÍTULO 4

Effects of environmental conditions on the regulation of plasma carotenoids and associated colouration in Black kites (*Milvus migrans*)

Effects of environmental conditions on the regulation of plasma carotenoids and associated colouration in Black kites (*Milvus migrans*)

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Short title: Influence of environmental conditions on carotenoid physiology

Key words: flood levels, raptor, senescence, signal expression, trade-offs

ABSTRACT

Animals that display carotenoid-based ornaments (e.g. brightly coloured integuments) often must face trade-offs in the allocation of these pigments between competing health and colour functions. To date, many studies have attempted to identify the ecological and internal determinants of these compromises, but frequent discrepancies in the results have precluded a clear understanding of the mechanisms behind the regulation of carotenoids. One potential reason is the fact that varying background environmental conditions may impose highly different constraints on individuals, which could either enhance or relax the different trade-offs, yet this aspect has received little attention so far. In this study, we examined whether inter-annual differences in marshland flood levels (drought vs high flooding) affected the strength or direction of the relationship between plasma carotenoid titres and several physiological and ecological variables in the black kite (*Milvus migrans*), a long-lived raptor ecologically linked to wetlands. Overall, plasma carotenoid levels were more elevated in wet than dry years, probably reflecting a higher availability of carotenoids in the environment. Plasma carotenoid levels were also higher in breeders compared to non-breeders, and correlated negatively with body condition, but only in years of drought, whereas males exhibited higher carotenoid levels than females only in years of high flooding. Likewise, seasonal trends in carotenoid levels differed under the two environmental scenarios, pointing to the background effect of desiccation dynamics on prey availability. In addition, tarsus coloration in wet years mirrored the age-structured improvements and senescent declines in reproductive success, survival and stress levels previously reported for this population, supporting the idea that the

honesty of carotenoid-based traits as indicators of differences in individual quality is maintained even when such differences are not evident in plasma carotenoid levels. Overall, our results highlight the importance of environmental conditions as potential, background constraints in carotenoid regulation.

INTRODUCTION

Carotenoids are probably best known among behavioural ecologists for their role in animal ornamentation and visual communication, since they confer most of the characteristic yellow, orange and red colours displayed by many animal species in integumentary tissue (e.g., feathers, scales, bare skin). Far less conspicuous, but equally important, is their function as physiological mediators in a range of health-related processes, acting both as potent stimulants of cellular and humoral components of the adaptive immune response (Bortolotti et al. 2000, Saino et al. 2003, Chew & Park 2004), and antioxidant agents involved in the protection of cell DNA, proteins and lipids from free radical attack (Pérez et al. 2008, Sindhu et al. 2010). In this context, animals are expected to accumulate as many carotenoids as possible in order to ensure that enough are available to fulfil their multiple competing functions. However, only plants, algae and some bacteria possess the necessary enzymes to synthesise carotenoids *de novo*, meaning that animals must necessarily acquire them from their diet, either directly as primary consumers, or indirectly by feeding on prey which have previously ingested and incorporated the pigments into their tissues. In the latter case, species at the upper levels of the food

chain, such as apex predators, may be particularly liable to suffer severe limitations in their average carotenoid intake (Negro et al. 2002, Casagrande et al. 2007), given that the concentration of these pigments becomes increasingly diluted at each step of the chain. For such species, life history theory predicts that individuals should give priority to the allocation of carotenoids towards maintenance functions over bright colour displays. Therefore, by extension, only healthy individuals or those with high foraging skills, capable of accumulating sufficient carotenoids either by capturing many prey or selecting those with a high carotenoid content, should afford to invest heavily in ornamentation without compromising survival. In this sense, carotenoid-based traits have been suggested to act as honest indicators of individual quality, which could be used by conspecifics in mate selection or intrasexual contests.

To date, a very large number of studies have placed their research focus on attempting to test the above predictions (e.g. Merilä et al. 1999, Hõrak et al. 2001, Casagrande et al. 2006). Still, the proximate factors responsible for the observed natural variation in circulating carotenoid levels and colour intensities remain often obscure. This is largely attributed to the frequent discrepancies in the results reported by different studies, even when considering the same species. One possible explanation is that the background environmental gradient within which the measurements are taken is too often ignored, despite the widely recognized potential of environmental variables to exert profound effects on many diverse aspects of animal physiology, including carotenoid regulation (No & Storebakken 1991, Török et al. 2007, Vergara et al. 2012a). For example, individual constraints faced at the time of acquiring these pigments may not solely rely upon the species' life-history feeding strategy (e.g. herbivore versus carnivore) or one's own foraging abilities, but can also depend on an array of external factors such as the availability

of carotenoid-containing resources present in an area (Negro et al. 2000), the number of competitors among which those resources must be shared (Vergara et al. 2012b), or the way in which climatic conditions affect their accessibility (e.g. vegetation under snow cover). On top of this, other factors like parasite prevalence or predation pressure may indirectly affect circulating carotenoid levels through their respective effects on infection status or stress physiology (since stress suppresses immune function and increases oxidative damage; Elftman et al. 2007, Constantini et al. 2011, Vergara et al. 2012b). The implication of this network of relationships is that the information content of certain carotenoid-based traits could change rapidly and markedly under different environmental situations. Thus, under favourable environments, where average body condition, health or circulating carotenoid concentrations might be expected to be significantly elevated, individuals could experience a relaxation in the allocation trade-off of carotenoids between ornamentation and immunocompetence in favour of the former, resulting in weaker visual differences between high and low quality conspecifics. This example highlights the importance of conducting studies seeking to unravel the way in which background environmental conditions affect the strength or direction of the relationship between phenotypic components of individual quality and levels of circulating carotenoids/carotenoid-based coloration. Such approach could improve our understanding of the proximate mechanisms behind signal expression, and help to explain the frequent inconsistencies reported by previous studies.

Temporary wetlands offer ideal settings for this purpose. One of the characteristic features of these habitats is that they undergo seasonal inundation-desiccation cycles which drive large-scale and often drastic changes in the quality of the environment within a relatively short period. As water bodies dry up, the size of submerged macrophyte communities is greatly reduced (Duarte et al. 1990),

producing a negative impact on primary productivity rates (and consequently on environmental levels of carotenoid precursors), that impinges upon all levels of the trophic chain. Moreover, decreasing water levels imply a reduction in the extension of flooded area, and thus of the physical holding capacity of the habitat, leading to an overall decline in both the total number of species and their abundance (i.e. lower food resources for predators; Newbrey et al. 2013). Superimposed on such seasonal trends, between-year differences in rainfall regimes can also result in marked inter-annual variations in maximum water levels (Davis & Ogden 1994, García & Marín 2006, Sergio et al. 2011a), giving rise to similarly contrasting ecological scenarios.

In light of the above, the first goal of the present study was to investigate the regulation of plasma carotenoid levels in a long-lived raptor under opposing environmental conditions. To this end, we sampled individuals from a population of black kites (*Milvus migrans*) dependent on Spain's largest natural wetland, in years that were characterised by either very high or very low spring flood levels (as a proxy of resource availability and overall habitat quality). Next, we: (1) compared average population-level concentrations of plasma carotenoids in "dry" versus "wet" years, predicting that these would be higher in the latter; and (2) explored potential relationships between carotenoid levels and a number of estimates of individual quality (e.g. body condition, body size, age and breeding status), as well as sampling date (to account for the progressive seasonal decline in water levels), under the two contrasting scenarios. In this case, we predicted that favourable environmental conditions (i.e. high flooding) would relax the constraints faced by kites at the time of acquiring carotenoids, thus resulting in weak differences in plasma levels between high and low quality individuals.

Following this line of argument, our second goal involved exploring whether such presumed relaxation in individual differences at the plasmatic level would also dilute the information content of carotenoid-based signals. In particular, we hypothesised that if the brightly coloured tarsi exhibited by kites are to be truly involved in intraspecies communication by acting as honest indicators of overall individual quality (as suggested by previous studies; Blas et al. 2013), internal regulation mechanisms should somehow prevent cheating by ensuring that variation in colour intensities reflects differences between high and low quality birds, even under scenarios of high food availability. To test this, we took colour readings on a subset of the individuals sampled in favourable (i.e. wet) years and examined variation in colour scores in relation to the same physiological and ecological variables as in the above analyses. This time, however, we predicted that if tarsus colour reliably conveys information on overall individual quality (i.e. body condition, foraging and fighting skills, social status, pathogen load…) we should find a positive and significant relationship with the variables that reflect those qualities. If, on the hand, tarsus colour intensity relies largely on passive diffusion processes, we would expect to find bright colorations across most individuals, resulting in no appreciable relationships with quality estimates.

MATERIALS AND METHODS

Study area and species

Black kites are medium-sized, migratory raptors with a life span of up to 28 years in the wild. Males and females exhibit similar plumage and body size characteristics, but differ in body mass, with females weighing ca. 15% more than males on average. European populations of the species spend the winters in Africa

and return to their natal grounds each spring, covering a total of nearly 6000km per year (Sergio et al 2014). Our study was conducted within Doñana National Park. This protected natural space, located on the right bank of the Guadalquivir river estuary (SW Spain), comprises a network of lagoons and temporary marshes of great ecological value. The hydrological regime of the system is largely dependent on the amount of autumn-winter rainfall (responsible for flooding the marshes every year between October and February), but also on spring-summer temperatures (which cause their drying up from February to around July; García & Marín 2006). Therefore, depending on annual climatic conditions, the extent of the marshes, as well as their hydroperiod can vary drastically from one year to the next. Both factors have been reported to exert a profound impact on the reproductive success of breeding kites and their nestlings' stress levels (Sergio et al. 2011a, Chapter 1 in this thesis). Moreover, since non-breeding individuals coexist in space with breeders and share similar foraging habitat preferences (Tanferna et al. 2013), these fluctuations are equally expected to impact on this less well studied sector of the population.

Black kites, like most raptors, do not display carotenoid-based plumage coloration, but instead exhibit yellow-orange coloured legs and ceres which show considerable intra- and inter-individual variation. In a recent study conducted on a sample of 127 black kites belonging to our study population (Blas et al. 2013), colour scores were found to differ significantly between breeders and floaters, but not with respect to sex or season, suggesting that carotenoid-based coloured traits in this species are not primarily involved in mate choice, but rather act as indicators of social status. In this study, we aimed to delve deeper into the communication function of colour expression in kites by testing the consistency of the above relationships under a favourable context, where the honesty of the information

conveyed to other conspecifics could be compromised by the fact that both high and low quality individuals may be able to accumulate sufficiently large quantities of carotenoids in plasma to afford investing heavily on the integument.

Field procedures

Black kites were captured each year between 2009-2012 through cannon-netting. All birds used in this analysis were originally ringed as nestlings and were thus of exact known age. For these, we measured tarsus length to the nearest 0.1 mm, wing and tail length to the nearest 1.0 mm and body mass to the nearest 5 grams. Tarsus colour was assessed by means of a portable CM-2600d spectrophotometer (Konica Minolta, Inc.). In order to avoid biased measurements caused by mud or dirt remains on the tarsi, we first cleaned the area of skin to be probed with water, and allowed it to air dry before taking reflectance readings (in duplicate). Next, we collected 0.5ml blood samples from the brachial vein and transferred the whole volume to heparinized tubes. These were stored in ice to prevent the oxidation of carotenoid molecules and the chemical degradation of blood proteins during the interval between sample collection and arrival at the laboratory facilities 2-3 hours later. Here, blood was centrifuged for 10 minutes at 1430g to allow the separation of cellular and plasma fractions, which were stored at -80°C until subsequent molecular sexing (Ellegren 1996) and plasma carotenoid quantification analyses (Bortolotti et al. 2000). Ring-reading sessions were conducted throughout the whole breeding season at all known territories and communal roosts, as part of an ongoing population monitoring program. Such information allowed us to reliably classify each of the birds as being either breeders or non-breeders in the year in which they were sampled.

Carotenoid quantification

The quantification of plasma carotenoid levels was conducted through a slight modification of the methodology described by Bortolotti et al. (2000). First, plasma samples were diluted with acetone in a 1:4 ratio (30µl and 120µl respectively). The mixtures were thoroughly vortexed and immediately transferred to a sonicating water bath at ambient temperature for 5 minutes to maximise homogenization. Next, samples were centrifuged at 1500g for a further 5 minutes. The resultant supernatant was collected in a new microtube (taking care not to disturb the remaining pellet during the process), air-dried using nitrogen gas (to prevent oxidation), and diluted again in 120µl of fresh acetone. The optical density of the resultant carotenoid-containing solution was determined using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA.) set at 450nm (the wavelength of maximum absorbance of lutein). Each sample was measured at least 3 times, and average values were used to calculate the concentration (in µg mL^{-1}) of carotenoids against a standard lutein curve.

Tarsus colour assessment

Output data from the spectrophotometer readings consisted of reflectance values at 10nm intervals within the 360nm-700nm range of the bird visible spectrum. Average reflectance (R) values for each wavelength (λ) were calculated for each bird from the replicate spectrophotometer readings. To quantify tarsus colour, we first calculated the three following colour variables (Andersson & Prager 2006): i) hue (H): here defined as the wavelength mid-way between the wavelengths of maximum and minimum reflectance, $(\lambda_{Rmax}+\lambda_{Rmin})/2$; ii) carotenoid saturation (S): defined as the amount of reflectance coming from the carotenoid part of the

spectrum expressed a proportion of the total spectral reflectance, $(R_{\lambda 700}-R_{\lambda 450})/R_{\lambda 700}$; iii) brightness (B): defined as the average spectral reflectance (i.e. the sum of reflectance values at each wavelength, divided by the total number of intervals). Then, we obtained an overall index of tarsus colour by combining these three measures into a single variable using a Principal Components Analysis (PCA). Principal component 1 (PC1) explained 67% of the total variance and showed high positive loadings for carotenoid saturation and hue, and negative loadings for brightness. Based on these values, we interpreted increasing tarsus colour PC1 scores to reflect the progression from pale yellow to intense orange colorations.

Statistical analyses

Tarsus, wing and tail length measurements were subjected to a PCA analysis, allowing extraction of a single variable (body PC1) representing overall structural size. The resultant body size scores explained 62.4% of the total variance and showed positive loadings for all three original variables. Body condition was estimated as the residuals of the ordinary least squares regression of body mass on body PC1, following Schulte-Hostdde et al. (2005). This analysis was conducted for males and females separately in order to control for sexual variation in mass. The level of inundation in each of the years of study (2009-2012) was estimated as the extent of flooded marshland at the beginning of the sampling period (March-April). For this purpose, we accessed the available cloud-free Landsat MSS, TM and ETM+ images for similar dates and compared inundation masks between years. On the basis of these comparisons, we built an "inundation index" where 2010 and 2011 were categorised as "wet" years, and 2009 and 2012 as "dry".

The first prediction, regarding the potential difference in average circulating carotenoid levels between years of drought and years of high flooding, was tested by means of a Student's t-test. All other predictions were tested using Generalised Linear Models (GLM). To explore whether the environmental context affected the way in which plasma carotenoid concentrations varied in relation to a number of potentially influential intrinsic and extrinsic factors, we constructed separate GLMs for birds sampled in dry (N = 52 individuals) and wet (N = 44) years. In both cases, carotenoid levels (log-transformed) were fitted as the response variable, sampling date, sampling date^2, body condition, age, age^2 and body size as continuous predictor variables, and sex and status (breeders vs non-breeders) as categorical predictors. Quadratic terms were included to explore non-linear patterns considered as plausible based on accumulated knowledge of our model system. In particular, we were interested in testing whether the early seasonal decline in water levels could be exerting an initially positive effect on kite carotenoid levels, by favouring the concentration of aquatic prey, followed by a subsequent negative effect as the remaining water pools eventually disappear (Kushlan et al. 1975, Chapter 1 in this thesis). On the other hand, age^2 was included to test for potential carotenoid declines associated to senescence (Velando et al. 2006, Sergio et al 2011b). During modelling, we detected two potential collinearity issues, the first involving the association between breeding status and age (non-breeders being significantly younger than breeders), and the other between body size and age (older birds being larger than younger birds). To deal with the first issue, we ran separate models to include either age + age^2 or status in the linear predictor. Whenever this approach resulted in two competing final models, we selected the one having the lowest Akaike´s Information Criterion (AIC) whenever $\Delta AIC>2$, or the most biologically plausible when $\Delta AIC<2$ (Burnham & Anderson 2002). The second issue was

resolved by using the residuals of body PC1 on age. Final models were derived following a backward stepwise procedure with significance set at $P \leq 0.05$.

The last set of analyses seeked to explore the potential of carotenoid-based colour traits to convey reliable information on individual quality under highly favourable environmental contexts (i.e. "wet" years). To this end, we constructed GLM models in the same way as described above, except for two aspects: 1) colour PC1 scores (N = 34) were fitted as the response variable; and 2) plasma carotenoid levels were fitted as an additional explanatory variable.

RESULTS

On average, plasma carotenoid levels were significantly higher in years of high flooding compared to years of drought (wet years: mean = 5.24 μg mL^{-1} ± 0.74 SE; dry years: mean = 2.97 μg mL^{-1} ± 0.40 SE; range =0.25 – 21.18 μg mL^{-1}; F = 1.73, P = 0.01, df = 94). Also, factors explaining individual variation in carotenoid levels changed between the two contrasting environmental conditions. In high-flooding years, carotenoid levels increased with sampling date (estimate = 0.03 ± 0.005 SE, F = 34.64, P <0.01; Fig. 1A), and were higher in males than females (males: mean = 5.93 μg mL^{-1} ± 1.01 SE; females: mean = 3.89 μg mL^{-1} ± 0.87 SE; estimate = -0.55 ± 0.24 SE, F = 5.55, P = 0.02; Fig. 2). On the other hand, in years of drought, carotenoid levels were negatively related to body condition (estimate = -0.005 ± 0.001 SE, F = 14.40, P <0.01), and were higher in breeders than non-breeders (breeders: mean = 4.02 μg mL^{-1} ± 0.75; non-breeders: mean = 2.15 μg mL^{-1} ± 0.34; estimate = -0.65 ± 0.27, F = 5.67, P = 0.02; Fig. 3). Interestingly, plasma

pigment levels varied quadratically along the season, increasing during the first half of the sampling period and subsequently declining (Fig. 1B), only the effect was marginally non-significant (estimate = -0.0004 ± 0.0002, F = 3.45, P = 0.07).

Tarsus colour scores in wet years correlated positively with plasma carotenoid levels (estimate = 0.08 ± 0.03 SE, F = 5.41, P = 0.03) and exhibited a quadratic relationship with age (estimate = -0.03 ± 0.01, F = 6.36, P = 0.02). More specifically, colour scores increased during the early- to mid-life stages (up to 11 years of age), and declined thereafter (Fig. 4).

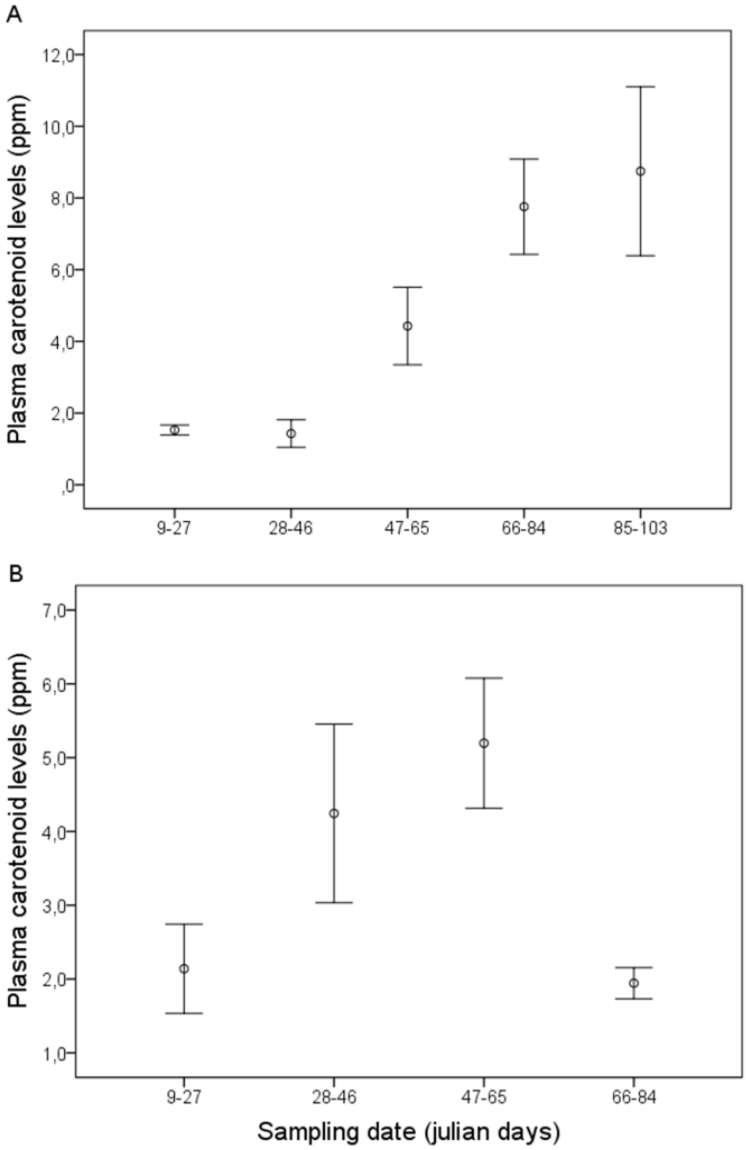

Fig. 1. Variation in mean plasma carotenoid levels of black kites with sampling date in (A) years of high inundation, and (B) years of drought. Bars represent ± 1 standard error.

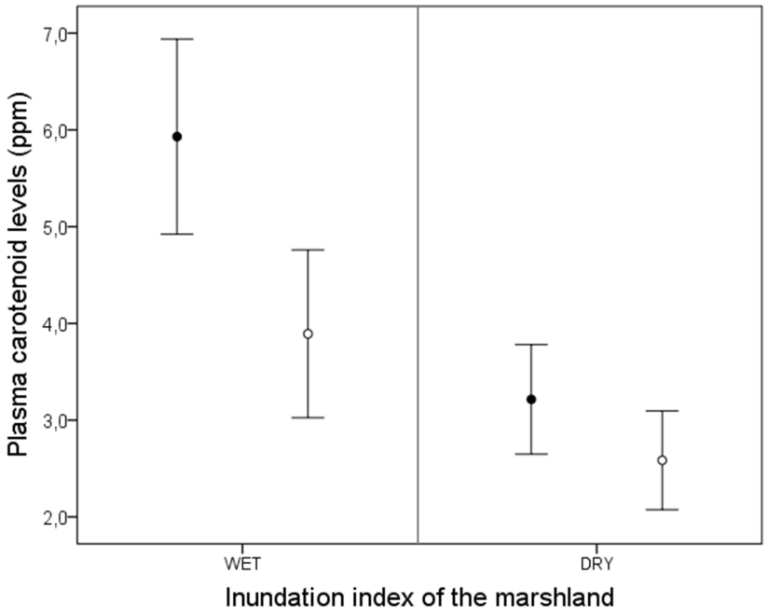

Fig. 2. Differences in mean plasma carotenoid levels between male (full circles) and female (empty circles) black kites, in years of drought (dry) and high inundation (wet). Bars represent ± 1 standard error.

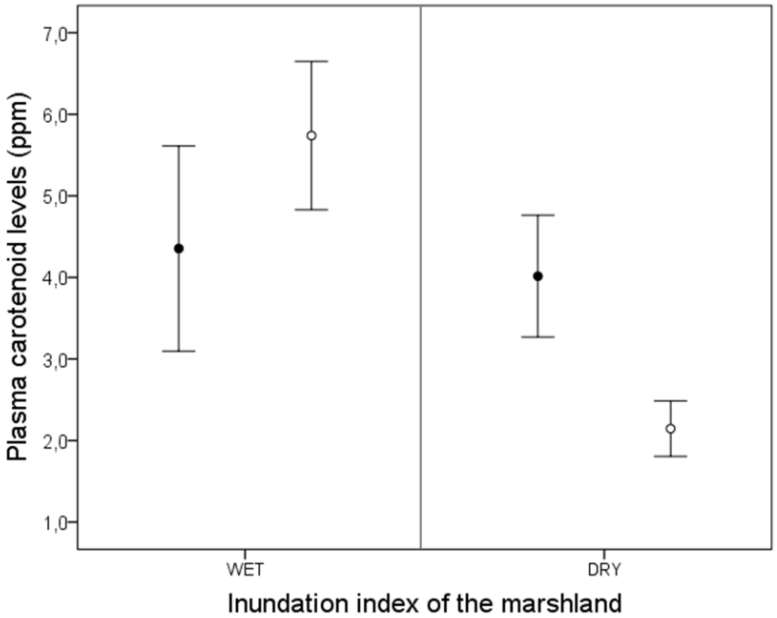

Fig. 3. Differences in mean plasma carotenoid levels between black kite breeders (full circles) and non-breeders (empty circles), in years of drought (dry) and high inundation (wet). Bars represent ± 1 standard error.

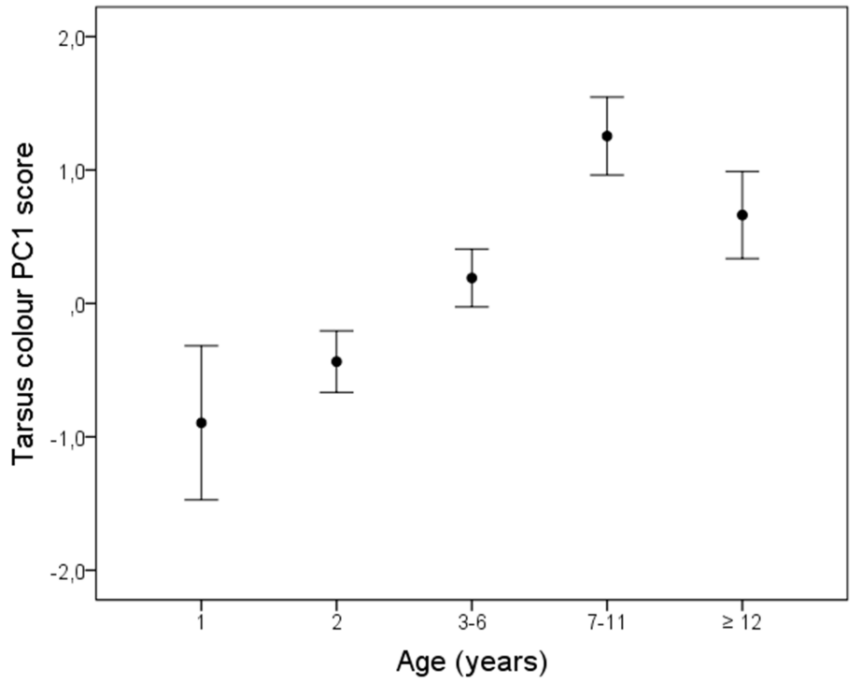

Fig. 4. Changes in the tarsus colour scores of Black kites with increasing age. Age groups follow the classification used by Sergio et al. (2001b, 2014) to identify the major life-history stages in the life cycle of this species. Bars represent ± 1 standard error.

DISCUSSION

Temporarily inundated wetlands undergo strong annual and seasonal fluctuations in flood levels which constitute a major fitness-driving force in many waterbird communities (Bildstein et al. 1990, Cèzilly et al. 1996, Desgranges et al. 2006), as well as in a large number of wetland-associated species, including black kites (Mooij et al. 2002, Sergio et al. 2011a, Newbrey et al. 2013, Chiavacci et al. 2014). However, our understanding of these fitness consequences has been largely focused on demographic parameters such as the number of breeding pairs or

average population reproductive success. In this sense, the results of the present study expand our knowledge of the functioning of these complex ecosystems by offering an eco-physiological perspective of the implications of fluctuating flood levels on other critical fitness-related components (i.e. health and intraspecific communication).

In agreement with our first prediction, average plasma carotenoid levels in black kites were higher in years of high flooding compared to years of drought. In general, low carotenoid levels are regarded either as an indication of poor health (since carotenoids are used up during infection to stimulate diverse components of the adaptive immune response and to neutralize free radical production; Saino et al. 1999), or of a weak potential to fight and recover from infection in the future (Baeta et al. 2008, McGraw & Ardia 2003). Our results, therefore, are consistent with the previously appreciated notion that low flood levels can impose severe ecological constraints on black kites, resulting in lower reproductive output (e.g. hatching and fledging success; Sergio et al. 2011a) and, as we here demonstrate, poorer physiological state. In the latter case, such constraints are likely related to the increased difficulty of obtaining sufficient carotenoids from the diet in dry years, when both primary productivity associated with macrophyte communities (which determines baseline environmental availability of carotenoids), and marsh prey numbers (which represent the major source of carotenoids for black kites) experience a drastic crash (Duarte et al. 1990, Newbrey et al. 2013).

Plasma carotenoid levels also varied between breeders and non-breeders, males and females, and in relation to body condition and sampling date. However, as predicted, both the strength and direction of these associations differed significantly depending on the environmental context. Breeders, for instance, exhibited higher plasma carotenoid levels than non-breeders but only in years of drought, possibly

reflecting the greater experience and, consequently, superior ability of the former to find and acquire resources during periods of food scarcity. This difference would be even further accentuated under the assumption that only high quality individuals (i.e. those potentially capable of procuring sufficient food to maintain themselves and their broods) make the decision to attempt breeding under unfavourable conditions, as seems to be suggested by the lower breeding densities recorded for this population in years of low flooding (Bildstein et al. 1990, Sergio et al. 2011a). At the same time, the reported pattern could also reflect different constraints in the carotenoid requirements of breeders and non-breeders. In this sense, breeders might be expected to be under a higher selective pressure to invest greater amounts of time and effort than non-breeders in procuring carotenoid-rich prey in carotenoid-poor environments in oder to boost their antioxidant defences and those of their nestlings, since both reproduction and development are metabolically active periods associated with large releases of harmful free radicals (Losdat et al. 2010). In turn, such constraint could allegedly force kites to face a trade-off between diet carotenoid and energy content; a situation which has already been reported in other species with exclusively carnivore diets. American kestrels, for instance, were found to circulate higher carotenoid levels when the density of voles (a poor source of carotenoids) was low within their territory, suggesting that, as the availability of their main prey declined, individuals switched to alternative, less energy-profitable prey (e.g. insects and passerine birds) but richer in carotenoid content (Bortolotti et al. 2000, Casagrande et al. 2007). Similarly, Montagu's harrier nestlings born in vole crash years exhibited worse body condition but developed more yellow carotenoid-based colorations in their tarsus skin and cere compared to nestlings born in vole peak years, when parents preyed almost exclusively on this small mammal (Sternalski et al. 2010). In line with these results, black kites in this study

showed higher levels of circulating carotenoids with decreasing body condition in years of drought. Such consistency in the direction of the association in these raptor species may indicate that the need to trade-off carotenoid content against caloric intake is a generalized trait among carnivores, likely exacerbated in carotenoid-limited environments. However, specific data on individual diet composition would be necessary to confirm this argument.

In contrast to the results above, sexual differences in plasma carotenoid titres were only evident in years of high flooding. The finding that males circulate more carotenoids than females is a common result in bird studies that is typically attributed, on the one hand, to the large quantities of pigment that mothers must transfer into the yolk of forming eggs (Negro et al. 1998, Isaksson et al. 2007), and on the other, to the fact that testosterone elevations in males during the mating season induce significant plasma carotenoid rises, which presumably serve to counteract the immunosuppressive action of the androgen (Blas et al. 2006). However, experimental studies in non-breeding birds have also reported significant differences in plasma carotenoid levels between males and females kept under the same diet (Bortolotti et al. 1996, Negro et al. 2001), implying that part of the observed variation between sexes could be under genetic or physiological control, and unrelated to breeding activities. Even so, the fact that these differences were obvious in wet years but absent in dry years was an *a priory* unexpected result. One possible reason for this pattern could be that males and females exhibit different physiological ceilings to the uptake of carotenoids from the gut, due to, for instance, differences in the plasma levels of carrier lipoproteins (i.e lower in females; Wallaert & Babin 1994, McGraw et al. 2006). In such case, significant differences between the two sexes would be more likely to develop under carotenoid-rich contexts, since males would be more capable of accumulating concentrations

beyond females' carrying capacity. Alternatively, sex-related differences in the regulation of carotenoids could be happening in the post-absorption stages. Once in the blood, carotenoid molecules are transported and deposited in a multitude of internal tissues like the retina, ovaries, kidneys and muscle, but especially fat stores (McGraw 2006). This aspect of carotenoid regulation could be particularly relevant for black kites, considering that females generally attain better body conditions than males and therefore might be expected to be able to draw larger amounts of carotenoids out of circulation for storage in adipose tissue. Under this assumption, sex differences would again be predicted to be larger in wet years, when abundance of aquatic prey should allegedly allow females to build considerably larger fat stores than males.

Interestingly, sampling date was the only variable that explained an important portion of the variation in the levels of plasma carotenoids of black kites in both dry and wet years. However, the direction of the association differed under each of the two environmental contexts in ways that suggest an important role of the desiccation dynamics of the marshland on carotenoid physiology. In years of drought, plasma pigment levels augmented between March and late-April, coinciding with the start of the desiccation period. In principle, receding water levels could initially benefit black kites by favouring the concentration of aquatic prey in increasingly smaller water-pools or, alternatively, because shallower water could facilitate the detection and capture of certain prey items such as fish and crustaceans (Kushlan et al. 1975, Bancroft et al. 2002). Later in the season, however, as the marsh continued to dry, carotenoid levels showed a marked decline, implying that, below a certain water threshold, prey abundance rapidly collapses. Along this line of thought, the finding that carotenoid titres in wet years increased across the whole range of sampling dates would simply reflect the fact

that marshland water levels were still above this minimum threshold on the last day of sampling. In contrast to these results, earlier studies with kestrels demonstrated steady seasonal declines in the average plasma carotenoid concentrations of captive individuals that had been maintained on an *ad libitum* carotenoid-rich diet (Negro et al. 1998). Such pattern could imply that birds are not under constant selective pressure to accumulate maximum carotenoid levels at all times, but rather that these may be internally regulated to match physiological requirements. However, our finding that plasma levels increased well into the rearing period in years of high inundation appears to suggest that such mechanism might only become apparent when access to carotenoids is completely unlimited, i.e. a condition that may rarely happen for raptors (and other animals) in the wild (Isaksson et al. 2007).

In black kites, variation in integumentary carotenoid-based coloration has been suggested to signal differences in social status and individual quality (Blas et al. 2013). Such property of coloured traits would be highly adaptive in this and other species where individuals are often involved in aggressive fights to compete for food resources, or to defend their territories against take-over or brood predation attempts (Sergio et al. 2011b). In this context, having a trait that communicates the quality of potential opponents beforehand would reduce the risk of becoming involved in highly mismatched fights that could result in serious or fatal injuries. However, in order to truly serve this purpose, the information conveyed by carotenoid-based traits should be consistent under all environmental contexts. In this study, tarsus colour scores in years of high flooding did not correlate with any of the variables that significantly explained variation in plasma carotenoid levels (i.e. sex and sampling date), suggesting that the allocation of carotenoid pigments to the body integument is largely an actively regulated process rather than one depending solely on simple passive diffusion (Casagrande et al. 2006). Moreover,

the reported lack of sexual and seasonal changes is consistent with previous results on the same population, reinforcing the suggestion that mate choice is most likely not the primary role of tarsus coloration in black kites. The reason behind this could be the highly monogamous nature of this species, since pairs typically establish long-term bonds which eliminate the need to select a new mate for many breeding seasons (Blas et al. 2013). Instead, colour scores correlated with individual age in a non-linear manner, involving a gradual progression from pale yellow to intense orange scores during the first 11 years of life, and a slight regression thereafter (≥12 years old). This pattern is consistent with the idea that, in black kites, carotenoid-based colours function as honest signals of the quality and social status of individuals even under the relaxed selection pressure given by resource- and carotenoid-rich environments. In long-lived species, individuals gain increasing experience and develop new skills through time, which help them to become better foragers and competitors. These abilities are well-known mediators of early- to mid-life age-structured improvements in a number of fitness and fitness-related components like recruitment, reproductive success, survival, migratory performance and stress levels, both in this population (Blas et al. 2009, Sergio et al. 2011c, Sergio et al. 2014, Chapter 3 in this thesis) and in many other vertebrate taxa (Forslund & Larsson 1992, Bowen et al. 2006, Balbontin et al. 2007). Likewise, such progressive improvement of essential skills with age could also promote an increasing investment of carotenoid pigments into integumentary tissue through several non-exclusive mechanisms: (1) higher foraging efficiency could allow individuals to accumulate sufficient carotenoids through the diet to fulfil both signalling and health functions, thus alleviating the trade-off between the two; (2) better fighting abilities could reduce the risk of suffering injuries, which could monopolize carotenoid-use to boost the immune system or to scavenge free radicals produced

during inflammatory responses (von Schantz et al. 1999, Cote et al. 2010); and (3) since chronic stress can induce immune suppression and free radial production, which, then again, consume carotenoids (Elftman et al. 2007, Constantini et al. 2011), older, more skilled and consequently less stressed individuals (Chapter 3 in this thesis) should be able to afford allocating more pigment into ornamentation (Mougeot et al. 2010). Following this line of argument, the decrease in tarsus scores exhibited by the oldest kites would imply a late-life decline in individual quality, which again agrees with previous reports of senescence-related declines performance in this population and other long-lived vertebrates (Weimerskirch 1992, Sergio et al. 2001c, 2014, Bowen et al. 2006).

In conclusion, we have demonstrated how different environmental contexts can influence the strength or shape of the relationship between plasma carotenoid levels and several intrinsic/extrinsic variables, thus offering a plausible explanation for the frequent discrepancies in the results reported by earlier studies. More specifically, we suggest that carotenoid availability may be a key environmental constraint which may either exaggerate or relax physiological differences in the foraging efficiency of high versus low quality individuals, variation in the dietary requirements of breeders versus non-breeders, or differences between the sexes. Furthermore, our results suggest that marshland desiccation dynamics alter seasonal trends in circulating carotenoid titres, potentially masking temporal fluctuations in the levels of internal carotenoid demands. Overall, the present study has served to underscore the importance of considering the whole range of environmental conditions experienced by populations in the wild, in order to obtain a clearer picture of the mechanisms and constraints affecting the internal regulation of these pigments. Importantly, the recorded inter-individual variation in tarsus colour scores during wet years perfectly mirrored previously reported lifelong

changes in reproductive success, survival probabilities, migration performance and stress levels, lending support to the idea that carotenoid-based coloured traits can function as reliable indicators of individual quality even under carotenoid-rich environments. Such feature opens the door to the use of colour estimates as a complement to more standard measures of the health or fragility of individuals or populations.

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SÍNTESIS

Aunque nacida hace más de 60 años, la eco-fisiología animal es a día de hoy una disciplina en pleno auge. Este reciente interés surge a raíz de la creciente necesidad de comprender el modo en que las variables ecológicas actúan sobre los distintos mecanismos fisiológicos para producir cambios en el fenotipo o conducta de los individuos, fuera del tradicional ambiente controlado del laboratorio. Pese a la indiscutible utilidad de estos estudios experimentales para sentar las bases teóricas de estas relaciones, lo cierto es que los resultados a menudo no son extrapolables a un contexto natural, donde los animales están expuestos a multitud de presiones simultáneas de distinta índole (bióticas, abióticas, sociales, físicas, etc.), o a especies no modelo, especialmente cuando estas exhiben estrategias vitales claramente divergentes. En consecuencia, el objetivo de la presente tesis ha sido el de avanzar en esta materia, llevando a cabo cuatro estudios en los que se ha investigado el efecto de diversos factores ambientales, sociales e intrínsecos en la regulación de glucocorticoides (GCs) y carotenoides en una especie no modelo (el milano negro, *Milvus migrans*), a lo largo de distintas etapas vitales y en condiciones naturales. Para ello, se han empleado además distintas técnicas de cuantificación de GCs, lo que ha permitido (en el caso de los capítulos 1 y 2), comparar el tipo de información obtenida con cada una de ellas y evaluar así las posibles ventajas de combinar varias técnicas. En esta síntesis, se discutirán los principales resultados de dichos estudios desde una perspectiva integradora, haciendo especial énfasis en sus implicaciones y limitaciones, y terminando con unas reflexiones sobre las posibles líneas de investigación a seguir en el futuro.

Efectos intrínsecos y socio-ambientales en la regulación de glucocorticoides en pollos

La secreción aguda de corticosterona (CORT) en respuesta a una perturbación tiene como función principal inducir cambios adaptativos en la fisiología y conducta de un individuo que le permitan afrontar y resistir la situación adversa o, por el contrario, eludirla lo antes posible. Sin embargo, cuando esto no ocurre, ya sea porque las respuestas no son lo suficientemente efectivas, o porque la perturbación se prolonga demasiado en el tiempo, se genera una situación de estrés crónico en donde los niveles elevados y sostenidos de CORT pierden su valor adaptativo y comienzan a ejercer un efecto nocivo sobre diversos aspectos fisiológicos del organismo (i.e. inmunosupresión, retrasos en el crecimiento y desarrollo, déficit de memoria, aumento del estrés oxidativo; Kitaysky et al. 2003, Elftman et al. 2007, Müller et al. 2009, Constantini et al. 2011), llegando incluso a afectar a la supervivencia (Blas et al. 2007). De manera análoga, cabría esperar que los pollos de especies no-precociales se mostraran particularmente propensos a sufrir los efectos perjudiciales del CORT crónico, puesto que al nacer, y durante gran parte de su desarrollo en el nido, carecen de la capacidad de realizar muchos de los ajustes fisiológicos y conductuales necesarios para hacer frente a los retos en su entorno (Blas & Baos 2008). No obstante, en la práctica, varios estudios llevados a cabo hasta la fecha en especies altriciales y semi-altriciales sugieren que los pollos de estas aves sincronizan la maduración/activación de su eje HPA con el desarrollo de sus sistemas fisiológicos y la progresiva adquisición de habilidades esenciales, reduciendo de este modo el riesgo de sufrir patologías asociadas a la secreción elevada y prolongada de CORT (Sims & Holberton 2000, Love et al. 2003; Blas et

al. 2005; Quillfeldt et al. 2009). En línea con estos resultados, tanto los niveles plasmáticos basales de CORT en pollos de milanos, como la respuesta máxima al estrés de una perturbación experimental, mostraron una clara ontogenia caracterizada por un aumento progresivo en ambos parámetros desde al menos la tercera semana de vida hasta el final del periodo de desarrollo en el nido. Por desgracia, las dificultades metodológicas encontradas a la hora de obtener muestras de sangre de los pollos más pequeños no permitieron determinar el grado de funcionalidad adrenocortical en el momento de la eclosión, cuando los pollos son más vulnerables y podrían, por tanto, beneficiarse de una supresión completa del eje HPA. En este sentido, la cuantificación de CORT en pluma tampoco ayudó a resolver esta cuestión, ya que en pollos de milano las plumas "definitivas" (i.e. el plumaje juvenil) comienzan a emerger alrededor de los 12 días de vida. Aun así, los resultados obtenidos ofrecen algunas pistas interesantes al respecto. Para empezar, los segundos pollos nacidos en nidadas múltiples mostraron, en general, niveles de CORT en pluma significativamente elevados en comparación con los de primeros pollos de la misma edad, quienes a su vez mostraron niveles superiores a los de los pollos de nidadas individuales. Tales diferencias sugieren: (1) que el entorno social del nido (i.e. pollo único vs. nidadas múltiples) afecta a la secreción de CORT, probablemente como consecuencia de las frecuentes agresiones entre hermanos que se dan en esta especie durante las primeras semanas de vida; y (2) que el coste energético de estas agresiones es mayor en los hermanos pequeños. En apoyo a esta teoría, las diferencias dejaron de ser apreciables en torno a los 35 días de edad, debido, principalmente, a la reducción gradual en los niveles de CORT de los pollos de nidadas múltiples, al tiempo que los de los pollos únicos se mantenían estables durante todo el desarrollo. Dicha dinámica vendría a reflejar la disminución en la frecuencia de agresiones reportada previamente en estos pollos a

partir de la tercera semana de edad, cuando la jerarquía entre hermanos comienza a asentarse (Viñuela 1999). En este contexto, los resultados de la presente tesis respaldan las conclusiones de una serie de estudios experimentales con ratones (Leshner et al. 1980), que sugieren que la activación adrenocortical aguda podría servir, entre otras cosas, para facilitar la sumisión, induciendo a los perdedores de encuentros agonistas a rendirse cada vez más rápido y evitar así una posible escalada de agresividad y el consiguiente riesgo de lesiones o muerte. Sin embargo, por otro lado, la conocida acción de CORT sobre las reservas corporales de grasa y la producción de glucosa (gluconeogénesis) podría servir también para aportar la energía necesaria para sustentar la intensa actividad física asociada a estas peleas. En conjunto, los indicios expuestos llevan a suponer que los pollos de especies fratricidas como el milano podrían requerir de cierta capacidad de activación adrenocortical nada más nacer, a fin de hacer frente a este tipo de retos ineludibles a la escala del nido. No obstante, sería interesante poder confirmar esta hipótesis, puesto que hasta la fecha no se han llevado a cabo estudios dirigidos a determinar el momento exacto en que el eje HPA adquiere funcionalidad en aves no-precociales. Por consiguiente, sugerimos que el próximo paso a seguir en esta dirección podría consistir en tratar de cuantificar niveles de CORT tanto en el plumón que cubre el cuerpo de estos pollos nada más nacer (proporcionando información acerca de la actividad adrenocortical durante el desarrollo en el huevo), así como en el segundo plumón que lo reemplaza a los pocos días (y que registraría, por tanto, una posible actividad adrenocortical post-eclosión).

Otro de los objetivos clave de esta tesis ha sido el de comparar el tipo de información obtenida mediante la cuantificación de CORT en plasma y en pluma. En este sentido, los niveles en pluma han demostrado ser más efectivos a la hora de detectar los principales retos a los que se enfrentan los pollos durante su desarrollo

(i.e. la rivalidad entre hermanos, las bajas temperaturas ambientales y la escasez de alimento), mientras que los niveles en plasma han servido, principalmente, para establecer el modo en que la carga alostática de estos individuos fluctúa de manera predecible tanto a corto como a largo plazo. En concreto, los niveles basales de CORT, al igual que la respuesta máxima al estrés de la captura, mostraron una marcada ritmicidad diaria, caracterizada por un pico en la secreción de hormona al principio del día, niveles mínimos por la tarde, y un nuevo aumento a última hora del día. Este patrón coincide con el observado anteriormente en ejemplares adultos y juveniles de otras especies precociales y no-precociales diurnas, y cuya función podría consistir en anticipar los sistemas internos a las demandas energéticas asociadas a los picos de actividad diaria (El-Halawani 1973, Joseph & Meier 1973, Rich & Romero 2001, Carere et al. 2003). Al mismo tiempo, los niveles basales de CORT mostraron también un aumento progresivo con la edad que podría reflejar el modo en que la carga alostática de los pollos se incrementa previsiblemente a medida que estos crecen y adquieren mayor coordinación y habilidad locomotriz, resultando en un aumento de la actividad (y por consiguiente, de las demandas energéticas). No obstante, debe tenerse en cuenta que en el caso de los análisis de CORT plasmático sólo se contó con pollos de más de 3 semanas de edad, por lo que la falta de asociaciones significativas con los factores socioambientales que sí mostraron relación con los niveles de CORT en pluma, podría ser la consecuencia de haber excluido los pollos más vulnerables a las variaciones externas. Así y todo, las diferencias en los resultados obtenidos en los capítulos 1 y 2 ponen de manifiesto la importancia de combinar varias técnicas para obtener información más completa e integrada de los cambios en la capacidad adrenocortical y los factores que pueden poner en riesgo la salud de las poblaciones.

Cambios en el perfil de actividad adrenocortical a lo largo de la vida

Los animales, a lo largo de sus vidas, deben enfrentarse a innumerables retos que ponen en riesgo su salud e incluso su supervivencia, y que suelen traducirse en un aumento en la secreción de GCs en el torrente sanguíneo. Algunos de estos retos se repiten año tras año de manera predecible, en asociación con determinadas etapas del ciclo vital o estaciones del año (p. ej., la migración, la reproducción o las bajas temperaturas invernales), mientras que muchos otros, en cambio, surgen de forma imprevisible en cualquier momento (p. ej., intentos de depredación, enfermedades o escasez de alimento). En general, a medida que los individuos avanzan en la vida, su capacidad de afrontar dichos desafíos tiende a mejorar progresivamente en función de la experiencia adquirida, la escalada de posiciones en la jerarquía social, y/o los retos inmunológicos superados. Prueba de ello es el típico aumento en la tasa de supervivencia y éxito reproductor asociado a la edad que se observa en muchas especies de vertebrados (Forslund & Larsson 1992, Pyle et al. 2001, Tavecchia et al. 2001). Sin embargo, pocos son los estudios que, hasta la fecha, han explorado los posibles cambios subyacentes en la actividad adrenocortical de animales silvestres, y menos aún en vertebrados de larga vida, a pesar del reconocido potencial de los GCs como posibles mediadores fisiológicos de la eficacia biológica (Kitaysky et al. 2001, Angelier et al. 2006; Heidinger et al. 2006, Blas et al. 2007). En este sentido, los resultados de la presente tesis han contribuido a ampliar el conocimiento actual de los procesos ontogenéticos en aves, aportando datos sobre las variaciones en los niveles de CORT en pluma (como reflejo del perfil de activación adrenocortical, y por consiguiente de la carga

alostática individual) a lo largo de toda la vida de una especie territorial sumamente longeva. En concreto, el análisis de estas variaciones reveló un importante efecto de la etapa vital (o categoría de edad) y del tamaño corporal. En el caso del primero, los niveles de CORT en pluma disminuyeron progresivamente desde el primer año de edad hasta los 7-11 años, pero mostraron un ligero aumento en edades posteriores (≥12 años). En cambio, los niveles de CORT mostraron una relación lineal y negativa con el segundo, lo que se traduce en una disminución de la carga alostática a mayor tamaño corporal. En conjunto, los dos resultados sustentan la idea expuesta arriba, y que propone que tanto el perfeccionamiento de habilidades esenciales como la escalada en el status social con el paso de los años permiten a los individuos evitar riesgos o afrontarlos más eficientemente (i.e. requiriendo una menor intensidad de activación adrenocortical). Asimismo, el aumento de CORT en las plumas de los individuos más longevos vendría a reflejar cómo la pérdida de aptitudes físicas y fisiológicas asociada a la senescencia vuelve a incrementar la vulnerabilidad de estas aves a los retos ambientales y sociales en las últimas etapas de la vida.

Otro aspecto importante a destacar de estos resultados es el notable paralelismo entre las variaciones ontogenéticas en los niveles de CORT en pluma, y las reportadas anteriormente para la supervivencia y el éxito reproductor en la misma población de estudio (Blas et al. 2009, Sergio et al. 2011a). Tal consistencia entre patrones apunta a un posible papel del eje HPA en la regulación de los cambios en la eficacia biológica de las aves a lo largo de las distintas etapas vitales. Por ejemplo, la secreción elevada de CORT durante los primeros y últimos años de vida podría inducir una mayor tasa de abandono de puestas o nidadas a través de una reducción en los niveles de la hormona prolactina (cuya función normal consiste en estimular y mantener los comportamientos parentales), como ya se ha

demostrado previamente en gaviotas tridáctilas (Angelier et al. 2009). Al mismo tiempo, el conocido efecto nocivo del estrés crónico sobre las defensas inmunitarias y el balance oxidativo podría resultar en un aumento del riesgo de contraer enfermedades (Elftman et al. 2007, Constantini et al. 2011), o de que estas sean más virulentas, en los grupos de edad más vulnerables a los retos ambientales.

Efecto de los niveles de inundación de la marisma en la regulación de los carotenoides plasmáticos

Los carotenoides son moléculas pigmentarias de naturaleza lipídica, con importantes funciones relacionadas con la salud y la expresión de señales en animales (Bortolotti et al. 2000, Saino et al. 2003, Blas et al. 2006, McGraw 2006). No obstante, sólo las plantas, algas y algunas bacterias poseen las enzimas necesarias para sintetizar los carotenoides *de novo*, mientras que los animales deben por fuerza adquirirlos a través de la dieta. Esto implica que para muchas especies sean un recurso limitado, especialmente para aquellas al final de la cadena trófica, puesto que la concentración de pigmentos en los tejidos corporales de las presas tiende a diluirse en cada eslabón de la cadena (Bortolotti et al. 2000, Sternalski et al. 2010). En tales casos, los individuos podrían verse en la necesidad de tener que comprometer el uso de los carotenoides para fines ornamentales en favor de su empleo en funciones fisiológicas que aumenten sus probabilidades de supervivencia (i.e. inmunoestimulación, reducción del estrés oxidativo). Bajo esta disyuntiva, cabría esperar que tan sólo los individuos más hábiles o experimentados a la hora de obtener recursos, o bien aquellos sanos y con un sistema inmunitario eficiente, pudieran permitirse destinar una buena parte de pigmentos a aumentar la

intensidad de sus rasgos ornamentales sin arriesgar su salud (Lozano 1994). De este modo, las diferencias de color entre distintos individuos podrían ser usadas por otros congéneres como señales honestas de la calidad de sus portadores.

Partiendo de este planteamiento, el objetivo del capítulo 4 de esta tesis se enfocó a explorar el posible efecto de las condiciones ambientales de fondo en la esperada relación entre calidad individual y niveles de carotenoides en plasma, así como su repercusión en la honestidad de las señales corporales basadas en estos pigmentos. Para ello, se muestrearon individuos adultos de milano negro en años caracterizados por unos niveles de inundación de la marisma extremadamente altos o, por lo contrario, extremadamente bajos. Estos dos contextos ecológicos representan calidades de hábitat radicalmente opuestas, tanto en términos de producción primaria (que, a su vez, dictamina los niveles ambientales de carotenoides), como de disponibilidad de presas acuáticas (menor cuantos más bajos). Prueba de esto último son las bajas tasas en el éxito reproductor de esta especie, o las marcadas elevaciones en los niveles plasmáticos de CORT en pollos durante los años de sequía (Sergio et al. 2011b, Capítulo 1 de esta tesis). En línea con estos resultados, los milanos mostraron, en general, niveles inferiores de carotenoides en sangre en años de poca inundación comparados con años de mucha agua, lo que podría deberse a una menor ingesta de presas y/o al hecho de que estas contuviesen concentraciones más bajas de los pigmentos en sus tejidos. También se encontraron diferencias en las relaciones entre los niveles plasmáticos de carotenoides y las variables examinadas dependiendo del contexto ecológico. Para empezar, se observaron diferencias significativas entre reproductores y no-reproductores únicamente en años de poca inundación, cuando los primeros lograron acumular alrededor del doble de carotenoides en plasma que los segundos. Otro dato a tener en cuenta es que los niveles de los reproductores apenas variaron

entre años de sequía e inundación, mientras que los de los no-reproductores llegaron casi a triplicarse. Aunque el estudio es meramente correlacional y carece de datos de dietas individuales, por lo que cualquier conclusión debe ser tomada con precaución, los resultados en principio parecen apoyar la teoría de que bajo condiciones ambientales poco favorables, sólo los individuos con más experiencia, mejores habilidades y mayor estatus social son capaces de obtener los suficientes recursos para mantener unos niveles de carotenoides elevados. Por otro lado, cabe la posibilidad también de que los reproductores se vean forzados a mantener dichos niveles incluso en ambientes pobres en carotenoides, a fin de compensar la alta tasa de producción de radicales libres asociada a actividades intensas y metabólicamente costosas como son los comportamientos reproductivos. En este supuesto, las diferencias reportadas vendrían a reflejar un mayor esfuerzo por parte de los reproductores para conseguir estos pigmentos, ya sea invirtiendo más horas de campeo o seleccionando presas con un alto contenido en carotenoides.

En cambio, las diferencias entre machos y hembras sólo se manifestaron en años de mucha inundación. Los motivos para esto podrían ser varios. Por una parte, los machos podrían tener mayor capacidad de absorber carotenoides desde el intestino gracias a un posible efecto de la testosterona en la movilización de lipoproteínas transportadoras, al igual que se ha observado en otras especies (Wallaert & Babin 1994, McGraw et al. 2006). En tal caso, las diferencias entre sexos podrían hacerse más evidentes en ambientes ricos en carotenoides ya que los machos deberían ser más capaces de acumular moléculas pigmentarias por encima del nivel de saturación de las hembras. Por otra parte, las diferencias podrían estar relacionadas con la deposición de los carotenoides en el tejido adiposo (McGraw 2006). En este sentido, las hembras podrían estar retirando un mayor volumen de carotenoides de la circulación para depositarlo en sus reservas de grasa, ya que en

esta especie, las hembras alcanzan condiciones corporales superiores a las de los machos. En tal caso, las diferencias cabrían ser de nuevo más acusadas en años de mucha inundación, ya que la abundancia de recursos permitiría a las hembras acumular mayores cantidades de grasa.

En conjunto, los resultados de este capítulo ponen de manifiesto la necesidad de contemplar e incorporar las condiciones ambientales presentes en el momento del muestreo en los análisis que buscan comprender los factores que pueden influir en la regulación de estos pigmentos, ofreciendo además una explicación plausible para las frecuentes discrepancias encontradas hasta la fecha entre varios estudios. Más allá de esto, las implicaciones para la conservación de esta población concreta de milanos podrían ser también importantes. Desde el punto de vista del momento actual que vivimos, donde el cambio climático es una realidad y los modelos predictivos apuntan a un aumento mayor de las temperaturas y estaciones cada vez más secas (IPPC 2013), el hecho de que los niveles de carotenoides en plasma de los milanos fuesen significativamente más bajos en años de sequía es un dato preocupante, ya que podría suponer un empeoramiento en la salud de la población a largo plazo. Sería, por tanto, de gran interés constatar el valor real del papel de los carotenoides en la salud de estas aves. Para esto, se propone la combinación de estudios hematológicos con el seguimiento de los individuos muestreados a lo largo de toda su vida, a fin de determinar la repercusión de las variaciones en los niveles plasmáticos de estos pigmentos en las probabilidades futuras de supervivencia.

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CONCLUSIONES

1) Los pollos de milano negro muestran un claro aumento en los niveles basales de corticosterona (CORT) plasmática, así como en la respuesta máxima al estrés experimental, desde al menos la tercera semana de vida hasta el final de su desarrollo en el nido. Estos patrones sugieren, respectivamente: (1) que las demandas energéticas (carga alostática) de los pollos aumentan gradualmente a medida que avanza su desarrollo, crecen en tamaño y ganan movilidad; y (2) que los pollos sincronizan la maduración/activación de la capacidad de respuesta del eje hipotalámico-pituitario-adrenal (HPA) con la progresiva adquisición de habilidades y conductas que facilitan hacer frente a posibles retos, reduciendo así el riesgo de sufrir los efectos nocivos de las elevaciones agudas y prolongadas en los niveles plasmáticos de CORT.

2) A pesar de esta atenuación asociada a edades tempranas, los pollos exhiben una marcada ritmicidad en la secreción de CORT similar a la observada en adultos de algunas especies precociales y no-precociales, y que se caracteriza por un pico en los niveles de CORT basales y en la respuesta máxima aguda a primera hora de la mañana, seguido de un descenso gradual hasta la tarde, y un nuevo aumento a última hora del día. Este patrón diario, postulado como un mecanismo para inducir un estado fisiológico apropiado para hacer frente a las demandas energéticas asociadas al comienzo del periodo activo, sugiere que cierto grado de activación del eje HPA es imprescindible para regular los procesos esenciales para la vida, incluso durante las etapas más sensibles del desarrollo. Por último, el resultado pone de manifiesto la necesidad de tener en cuenta esta importante fuente de variación en estudios con pollos no-precociales de todas las edades.

3) Las variaciones en los niveles de CORT en plumas de pollos de milano negro sugieren que los principales retos a los que se enfrentan estas aves durante su periodo de desarrollo en el nido son las interacciones agresivas entre hermanos, las bajas temperaturas ambientales, y la falta de alimento. Más allá del valor ecológico de estos datos, los resultados de esta tesis demuestran el potencial de las plumas para reflejar el efecto de factores de distinta índole (sociales, ambientales e intrínsecos) sobre la fisiología de un individuo, contribuyendo de este modo a reforzar la idea de que las plumas actúan a modo de registros históricos de la actividad adrenocortical experimentada durante su crecimiento.

4) Los análisis de CORT en plasma y en pluma aportan informaciones muy distintas pero complementarias sobre el desarrollo de la función adrenocortical y los retos homeostáticos y energéticos que afronta un ave en distintos momentos de su vida y a lo largo de distintas escalas temporales. Queda demostrada, por tanto, la importancia de combinar ambas técnicas para obtener una visión más global e integrada del estado de salud de las poblaciones, así como de factores que pueden suponer un riesgo para estas, especialmente en estudios enfocados a diseñar medidas de conservación para especies en peligro.

5) Las variaciones en los niveles de CORT en pluma a lo largo de las distintas etapas de vida en milanos adultos sugieren una reducción gradual en la carga alostática de estas aves con el paso de los años (posiblemente asociada al perfeccionamiento de habilidades esenciales y a la escalada de posiciones en la jerarquía social), seguido de un aumento en la vulnerabilidad a los retos ambientales en los individuos más longevos (reflejando la pérdida gradual de aptitudes asociada a la senescencia). Por

otra parte, el notable paralelismo entre los niveles de CORT y los cambios ontogenéticos en la supervivencia y éxito reproductor de esta misma población apuntan a la contribución de este mecanismo fisiológico en la determinación de la eficacia biológica en esta especie. En conjunto, los resultados del capítulo 3 resaltan el potencial de esta técnica no-invasiva como herramienta complementaria en estudios de conservación para identificar las etapas o segmentos de la población más vulnerables de forma rápida y fiable.

6) Las condiciones ambientales de fondo (i.e. el contexto ecológico) en el momento de un muestreo son un factor importante a tener en cuenta a la hora de explorar los factores (internos y externos) que influyen en la regulación de los niveles plasmáticos de carotenoides. En esa tesis, la relación esperada entre calidad individual y concentración de pigmentos en plasma sólo se manifestó en años de sequía, cuando el número de presas acuáticas (y por consiguiente, el acceso a carotenoides), se reduce de manera drástica. Del mismo modo, cualquier otra variable ambiental con capacidad de limitar la disponibilidad de carotenoides, o de inducir un aumento en su consumo, podría igualmente afectar la regulación de estos pigmentos en función de sus parámetros. Esto ofrece, por tanto, un motivo plausible para las frecuentes discrepancias en los resultados de distintos estudios.

7) Al contrario que los niveles de CORT en pluma, la coloración del tarso en milanos muestra un aumento en la intensidad de pigmentación con la edad durante los primeros 11 años, pero disminuye en edades posteriores, cuando las aves comienzan a mostrar signos inequívocos de senescencia (i.e. descensos en la tasas de supervivencia y éxito reproductor). Este patrón concuerda con el esperado en un

carácter cuya función se ha sugerido que podría consistir en informar a otros conespecíficos sobre la calidad de un individuo. Más importante si cabe es el hecho de que esta variación se observa en años en los que las diferencias en la calidad individual a nivel plasmático (en términos de la concentración de carotenoides) no son evidentes, lo que sugiere que tanto las demandas internas como los mecanismos endógenos que regulan la incorporación de carotenoides al tegumento, mantienen la honestidad de la información de la señal más allá de la disponibilidad de estos pigmentos.

OTRAS PUBLICACIONES

Listado de publicaciones en las que he participado durante el transcurso de la realización de esta tesis doctoral.

1) Alcaide, M., López, L., Tanferna, A., Blas, J., Sergio, F., & Hiraldo, F. (2010) Simultaneous analysis of multiple PCR amplicons enhances capillary SSCP discrimination of MHC alleles. Electrophoresis, 31:1353-1356.

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